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THE CONTROL OF PLASTID PIGMENT FORMATION BY A VIRESCENT GENE, PALE-YELLOW-1, OF MAIZE[1]

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The effects of certain environmental factors upon the phenotypic expression of a single-gene mutation, pale-yellow-1, of maize have been described (13). In brief, the observations showed that the mutant seedling is initially uniform pale-yellow in color when grown in either light or darkness. However, when exposed to light at 25° C the mutant seedlings become green in color approximately 8 days after planting. Plastid size and number, type of culture media used, length of previous exposure to light and the presence of the endosperm are not factors in this delayed appearance of pigments.

This paper will present quantitative information on the pigment content and pigment changes which occur in the mutant, pale-yellow-1, of maize. In particular, an attempt will be made to establish the relationship of the mutant gene to the accumulation of specific carotenoid pigments and the chlorophylls. This will be done in order to determine the basis for the differences in color between the mutant and its normal sibling (13). In addition, it is hoped that some information about the biochemical relationship of the carotenoids to the chlorophylls will be obtained from these observations.

METHODS

The plants used in this study were germinated from seeds which were found to produce seedlings in a ratio of 3 normals to 1 mutant. All seedlings were grown in a constant temperature chamber at 25 ± 0.5° C. Light (700 ft-c) was supplied by 13 slimline fluorescent lamps labeled 4500° white. A description of the culture methods, light source and the constant temperature chamber has been reported (13).

Samples of leaf material were analyzed for flavoxanthin c, violaxanthin b, lutein, beta carotene, neo-beta carotene, chlorophyll a, chlorophyll b, and protochlorophyll a. Neo-beta carotene and beta carotene were the only carotenes found in the leaf material and flavoxanthin c, violaxanthin b, and lutein accounted for over 90 % of the total carotenol content. A detailed description of the methods used to make the pigment analyses has been presented (11). In brief, the analyses were made as follows. Fifteen leaves were removed from 15 different plants and divided into three samples of 5 leaves each. One leaf punch was taken from the distal 5 cm of each leaf with a 7 × 52-mm steel die and each sample of 5 leaf punches was analyzed for carotenoids or chlorophylls. When the samples were analyzed for carotenoids, the pigments were extracted with methanol, the chlorophylls saponified and the carotenoids transferred to a mixture of ethyl ether and petroleum ether. The carotenols were chromatographically resolved into individual components on a magnesia column, while the carotenes passed through the column and were recovered as a binary mixture in the perculate. Samples analyzed for chlorophylls were extracted with methanol and the chlorophylls transferred to petroleum ether. The chlorophylls were separated from the carotenoids and resolved into chlorophyll a and chlorophyll b by chromatographing the petroleum ether solution on a starch column. Protochlorophyll a was extracted from the leaf samples and chromatographically isolated by the method of Koski and Smith (12). The amounts of chlorophyll a, chlorophyll b, protochlorophyll a, lutein, violaxanthin b, and flavoxanthin c were calculated from the absorbance, at their respective maxima, and the specific absorption coefficients given by Zscheile and Comar (17), Strain (14) and Koski and Smith (12). The amounts of beta carotene and neo-beta carotene were calculated from the absorbance of the binary solution by the method of Beadle and Zscheile (2).

EXPERIMENTAL AND RESULTS

PIGMENT CONTENT OF DARK-GROWN MUTANT AND NORMAL SEEDLINGS: When grown at 25° C, 7-day-old dark-grown mutant seedlings are pale-yellow in color, while dark-grown normal seedlings are bright-yellow in color. This observation suggests that the concentration of carotenoids in the mutant seedlings is less than in the normal seedlings. However, it does not indicate how much less carotenoid is present and which carotenoid pigments are affected. Therefore, the pigment content of 7-day-old dark-grown mutant and normal seedlings was determined. The results are shown in table I. In the mutant seedlings the concentrations of flavoxanthin c, lutein, violaxanthin b, and protochlorophyll a are less than in the normal seedlings, while the concentrations of neo-beta carotene and beta carotene are the same in both types of seedlings.

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TABLE I

PLASTID PIGMENT CONTENT OF 7-DAY-OLD LIGHT-GROWN AND DARK-GROWN MUTANT AND NORMAL CORN SEEDLINGS

PIGMENT	LIGHT-GROWN SEEDLINGS		DARK-GROWN SEEDLINGS	
	NORMAL	MUTANT	NORMAL	MUTANT
	μgm pigment/gm dry wt of leaf			
Flavoxanthin c	110 ± 8.0*	62 ± 5.0	130 ± 6.2*	73 ± 3.0
Lutein	432 ± 25.0	206 ± 9.0	490 ± 14.0	247 ± 6.0
Violaxanthin b	67 ± 6.0	42 ± 4.0	184 ± 4.0	107 ± 5.0
Beta Carotene	514 ± 29.0	74 ± 3.0	81 ± 4.1	82 ± 6.0
Neo-beta Carotene	126 ± 1.4	36 ± 1.0	75 ± 3.2	79 ± 2.0
Protochlorophyll a	...	...	55 ± 9.0	6 ± 4.0
Chlorophyll a	5760 ± 180.0	0	...	...
Chlorophyll b	840 ± 23.0	0	...	...

* Mean plus-or-minus the standard error of the mean. The values are the means of three analyses. Each analysis was carried out on a pool of 5 leaf samples.

PIGMENT CONTENT OF LIGHT-GROWN MUTANT AND NORMAL SEEDLINGS: When grown in the light at 25° C 7-day-old mutant seedlings are pale-yellow in color and their normal siblings are deep-green in color. The deep-green color of the normal sibling masks the color due to the carotenoids, making it impossible to visually compare the carotenoid content of the mutant to that of the normal seedlings. However, quantitative, chromatographic pigment analyses of both normal and mutant seedlings reveal that the carotenoid content, as well as the chlorophyll content, of the mutant is different from that of the normal seedling. The pigment content of 7-day-old mutant and normal seedlings, grown in the light at 25° C, is shown in table I. As in dark-grown plants, the flavoxanthin c, lutein, and violaxanthin b content of the mutant seedlings is less than that of the normal seedlings. However, in contrast to the dark-grown material, the light-grown mutant plants also contain less neo-beta carotene and beta carotene than do the light-grown normal seedlings. The normal seedlings also contain large amounts of chlorophyll a and chlorophyll b, while these compounds are not detectable in the light-grown, 7-day-old mutant plants. It is also noteworthy that the amounts of carotenols found in both mutant and normal seedlings is less in light-exposed than in dark-grown plants. This decrease in the carotenol content in the light-exposed plants is due mainly to a large decrease in violaxanthin content.

PIGMENT CHANGES IN DARK-GROWN AND LIGHT-EXPOSED MUTANT AND NORMAL SEEDLINGS: Mutant seedlings grown in the light at 25° C begin to turn green in color when they are about 8 days of age and soon they become phenotypically indistinguishable from the normal seedlings. If accumulation of the carotenoids, as well as the chlorophylls, is controlled by the mutant gene it would seem reasonable to expect the carotenoid content of the mutant seedlings, as well as the chlorophyll content, to increase when the seedlings are about 8 days of age. To test this hypothesis two experiments were carried out.

In the first experiment mutant and normal seedlings were grown in darkness for 14 days and carotenoid determinations were made from samples taken every 24 hours, starting with 6-day-old seedlings. Protochlorophyll a analyses were also made on 6- and 10-day-old seedlings. In the second experiment, mutant and normal seedlings were grown in darkness at 25° C for 6 days and then exposed to continuous light. Carotenoid and chlorophyll determinations were made from samples taken 0 and 6 hours after exposure to light and thereafter at 24-hour intervals. In each experiment the changes in the components within each class of pigments (carotenols, carotenes and chlorophylls) followed similar courses. Therefore, the data are shown in figures 1 and 2 as total carotenols (flavoxanthin c, violaxanthin b, and lutein), total carotenes (neo-beta carotene and beta carotene) and total chlorophylls (chlorophyll a and chlorophyll b).

The data from the first experiment are shown in figure 1. Six days after planting, the carotenol content of dark-grown mutant seedlings is 60 % and the protochlorophyll a content 10 % of that found in the normal seedlings, whereas the carotene content of the mutant seedlings does not differ from that of the normal seedlings. The carotenol content of the dark-grown normal seedlings increases between the sixth and eighth days after planting and thereafter it declines. On the other hand, the carotenol content of the dark-grown mutant seedlings remains the same between the 6th and 8th days after planting and then it slowly increases. In both dark-grown mutant and dark-grown normal seedlings the carotene content increases between the sixth and eighth days after planting and thereafter it slowly decreases. The protochlorophyll a content of dark-grown normal plants does not change between the sixth and tenth days after planting, whereas in dark-grown mutant seedlings it increases. Thus, in dark-grown mutant seedlings both carotenols and protochlorophyll a begin to increase at the time the mutant block is broken, however an increase in carotene content does not occur at this time. In fact, the pattern of carotene accumulation in the mutant seedlings is almost

identical to that in the normal seedlings. This suggests that the mutant gene controls the formation of the protochlorophyll a and the carotenols, but not the carotenes.

Pigment changes in plants exposed to light seven days after planting are shown in figure 2. Within 5 hours after exposure to light, appreciable quantities of chlorophyll are found in normal plants; whereas no chlorophyll is detectable in light-exposed mutant seedlings until after 44 hours of exposure to light (approximately 8 days after planting). However, once chlorophyll is formed in the mutant its accumulation follows a pattern similar to that of the normal, and the maximum chlorophyll content is comparable to the maximum amounts found in the normal seedlings. This was true for both chlorophyll a and chlorophyll b. Upon exposure of the dark-grown plants to light there is a decrease in the carotenol content of both mutant and normal seedlings. As indicated earlier this decrease in carotenol content is mainly due to a large decrease in the concentration of violaxanthin b. The carotenol content of the normal seedlings decreases for about 98 hours and then slowly increases. In the mutant seedlings the carotenol content decreases for the first 47 hours after exposure to light and then it increases. The carotene content of normal seedlings increases within 6 hours following exposure to light and continues to increase in a manner paralleling chlorophyll formation for the next 66 hours, thereafter it remains relatively constant. On the other hand, the carotene content of the mutant seedlings decreases during the first 29 hours after exposure to light and no increase is evident until after 47 hours of exposure to light (8 days after planting). Once the carotene content of the mutant begins to increase, its accumulation, as in the normal seedlings, parallels that of chlorophyll and the carotene content becomes comparable to that of the normal seedlings.

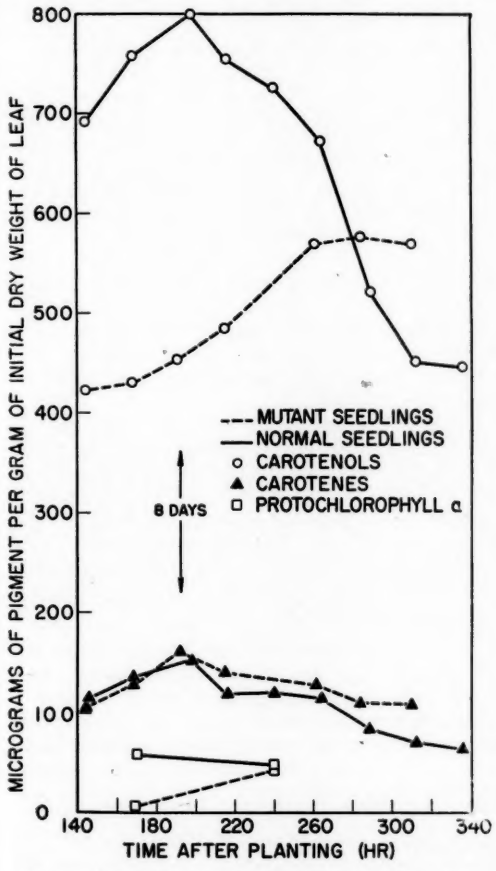

FIG. 1. Carotenol, carotene and protochlorophyll a accumulation in dark-grown mutant and normal corn seedlings. Determinations were started 6 days after planting. Carotenol content is the sum of lutein, flavoxanthin c and violaxanthin b. Carotene content is the sum of beta and neo-beta carotene.

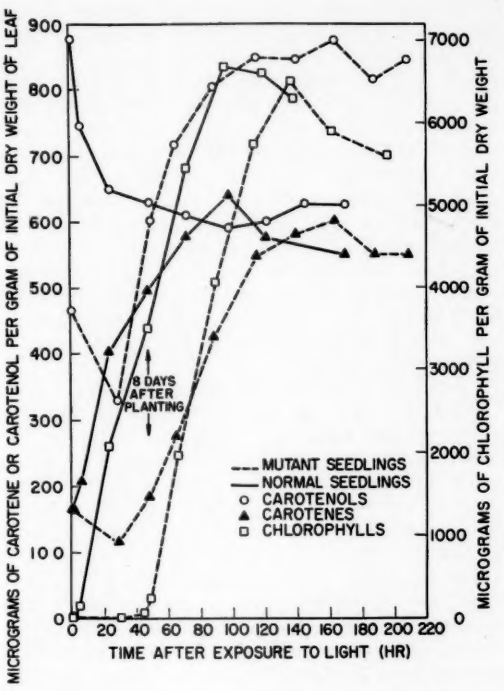
MICROGRAMS OF CAROTENE OR CAROTENOL PER GRAM OF INITIAL DRY WEIGHT OF LEAF
MICROGRAMS OF CHLOROPHYLL PER GRAM OF INITIAL DRY WEIGHT
TIME AFTER EXPOSURE TO LIGHT (HR)
MUTANT SEEDLINGS
NORMAL SEEDLINGS
CAROTENOLS
CAROTENES
CHLOROPHYLLS
8 DAYS AFTER PLANTING

FIG. 2. Carotenol, carotene and chlorophyll accumulation in light-exposed mutant and normal corn seedlings. Seedlings were grown in darkness for 6 days and then exposed to continuous light. Determinations were started 6 days after planting. The carotenol content is the sum of flavoxanthin c, violaxanthin b and lutein. The carotene content is the sum of beta and neo-beta carotene and the cholorophyll content is the sum of chlorophyll a and chlorophyll b.

It thus appears that the light-exposed mutant plants cannot begin to accumulate carotenes, carotenols or chlorophylls in amounts comparable to those found in light-exposed normal seedlings until approximately 8 days after planting. This implies that in light-exposed mutant seedlings the mutant gene controls the formation of the carotenols, carotenes and chlorophylls. However, if this is the case, carotenols and carotenes should not be present in the mutant leaves prior to 8 days after planting. It seemed possible that the endosperm might provide precursors for carotenoid and chlorophyll formation, which lie beyond the mutant block. Such precursors could readily give rise to the amounts of carotenoids and protochlorophyll a found in the mutant prior to 8 days after planting.

Pigment Content of Endospermless Dark-Grown Mutant and Normal Seedlings: Embryos were dissected from corn seeds and grown in the dark at 25° C on a sterile mineral solution (6) to which 0.15 mole of glucose per liter and 0.75 % agar were added before sterilization (10, 15). Intact seeds were germinated on the same medium to serve as controls. Plants developing from the excised embryos were small and had elongated leaves. In general, they resembled etiolated oat seedlings. The mutant plants were cream-white in color, while the normal plants were pale-yellow in color. Eight days after transfer of the embryo to the culture medium, the entire first and second leaf blades were analyzed for carotenols, carotenes and protochlorophyll a. The results of the analyses are given in table II.

Protochlorophyll a was not detected in the leaves of endospermless mutant seedlings, but it was present in unreduced amounts in the leaves of the endospermless normal seedlings. The carotene content of the endospermless mutant and normal seedlings was less than that of plants grown from intact seeds, and on both a percentage and absolute basis the reduction was the same in mutant and normal seedlings. The carotenol content of the endospermless mutant and normal seedlings was also less than that of plants grown from intact seeds and on an absolute basis the reduction was the same in both mutant and normal plants. However, on a percentage basis the reduction in the mutant seedlings was 92 %, while in the normal it was only 48 %. These data indicate that there is an endosperm factor that contributes to carotenoid and protochlorophyll a production in maize seedlings. Apparently, in 8-day-old mutant seedlings, carotenol production is almost completely dependent upon this endosperm factor and protochlorophyll a production is completely dependent upon it. In contrast, carotene production in the mutant, as in the normal, is only partially dependent upon this endosperm factor.

Discussion

In this discussion the carotenols (flavoxanthin c, violaxanthin b and lutein) will be considered as a group, since the changes in the individual carotenols paralleled each other and they are all hydroxylated carotenes (16). Beta carotene and neo-beta carotene will also be considered as a group, as the changes in neo-beta carotene and beta carotene followed the same pattern and they are isomeric hydrocarbons.

It is evident from the pigment analyses that the mutant seedling is different in color from the normal seedlings because of a deficiency in certain plastid pigments. When grown in darkness mutant seedlings are pale-yellow, whereas normal seedlings are bright-yellow. This is due to the decreased carotenol content of the mutant seedlings. Mutant seedlings grown in the light lack chlorophyll and contain less carotenols and carotenes than do normal seedlings. Thus they are pale-yellow in color while normal seedlings are green in color. Dark-grown, endospermless mutant seedlings are cream-white in color as contrasted to pale-yellow endospermless normal seedlings. This is because the endospermless mutant seedlings contain almost no carotenols.

Frank (8, 9) and others have suggested that the carotenoids and the phytyl portion of the chlorophyll molecule probably arise from colorless precursors common to both. On the basis of the unitary action of the gene (3–5), genetical evidence also indicates that the carotenoids and chlorophylls are closely interrelated in their biosynthesis, since many single genes are known to affect the presence of the carotenoids as well as the chlorophylls.

Table II

Plastid Pigment Content of Endospermless, 8-Day-Old, Dark-Grown Mutant and Normal Corn Seedlings

Pigment	Normal		Mutant	
	With endosperm	Without endosperm	With endosperm	Without endosperm
	μgm pigment/gm fresh wt of leaf			
Flavoxanthin c	6.4 ± 0.47 *	3.0 ± 0.50	3.3 ± 0.20	0.30 ± 0.10
Violaxanthin b	8.5 ± 0.19	4.9 ± 0.65	4.5 ± 0.40	0.40 ± 0.10
Lutein	31.0 ± 0.90	16.0 ± 0.68	16.5 ± 0.40	1.2 ± 0.10
Beta Carotene	3.2 ± 0.14	1.0 ± 0.31	3.1 ± 0.11	1.2 ± 0.11
Neo-Beta Carotene	1.8 ± 0.10	0.45 ± 0.10	1.5 ± 0.01	0.51 ± 0.02
Protochlorophyll a	2.4 ± 0.10	1.9 ± 0.20	< 0.50	0

* Mean plus-or-minus the standard error of the mean. The values are the means of three analyses.

In the present study the action of a single gene upon the accumulation of chlorophylls and carotenoids was investigated. Although the data do not indicate which step in the pathway of plastid pigment synthesis was affected by the mutant gene, they do provide information about the position of the mutant block relative to the production of carotenes, carotenols and chlorophylls.

During the first 8 days after planting, the mutant seedlings are devoid of chlorophyll when grown in the light, and when grown in darkness they contain only a very small amount of protochlorophyll a. This might imply that the mutant seedling is unable to accumulate chlorophyll because of an excessive destruction of protochlorophyll a. However, when endospermless mutant seedlings are grown in darkness they are devoid of protochlorophyll a. Therefore, it appears more likely that the protochlorophyll a found in the dark-grown mutant is derived from an endosperm factor which is independent of the mutant gene and that there is a block in chlorophyll synthesis prior to the formation of protochlorophyll a. The pigment analyses also show that, during the first 8 days after planting, the carotenol content of the mutant is always less than that of the normal. Although 7-day-old dark-grown mutant seedlings contain a relatively large amount of carotenol, 8-day-old dark-grown endospermless mutant seedlings contain virtually no carotenols. This strongly suggests that the carotenols found in the mutant seedlings, during the first 8 days after planting are derived from an endosperm factor. This endosperm factor appears to be independent of the mutant gene, since it contributes equally to carotenol synthesis in both the mutant and normal seedlings.

Additional evidence for the control of both chlorophyll and carotenol production by the mutant gene is found in the unique reversion of the mutant seedlings to a normal phenotype. About 8 days after planting, the mutant block is apparently broken and both dark-grown and light-exposed mutant seedlings begin to accumulate carotenols and chlorophylls (or protochlorophyll a). This would be expected if the mutant gene blocks carotenol and chlorophyll formation at a common point in their synthesis.

The accumulation of the carotenes does not appear to be under the control of the mutant gene. In dark-grown mutant plants the carotene content is equal to that of the normal seedlings and the pattern of carotene accumulation is the same as in the normal seedlings. Likewise dark-grown, endospermless mutant seedlings contain as much carotene as dark-grown, endospermless normal plants. Thus it appears that carotene is synthesized by the mutant seedlings. On the other hand, prior to 8 days after planting, normal seedlings exposed to light contain much more carotene than do mutant plants. This implies that in light-exposed mutant seedlings the synthesis of carotenes, as well as chlorophylls and carotenols, is controlled by the mutant gene. However, the control of carotene synthesis by the mutant gene in light-exposed mutant seedlings, may be an indirect effect. Bandurski (1) has indicated that photosynthetic products are necessary for the production of large amounts of carotene. The exposure of mutant seedlings to light, prior to eight days after planting, does not give rise to chlorophyll synthesis and thus the products of photosynthesis are not available for increased carotene production. Therefore it seems likely that the mutant gene blocks the synthesis of the chlorophylls (or protochlorophyll a) and the carotenols, while the synthesis of carotenes, in light-exposed mutant seedlings, is indirectly affected by a lack of photosynthetic products due to the inability of the mutant seedlings to synthesize chlorophyll.

If the phytyl portion of the chlorophyll molecule and the carotenoids arise from common precursors it seems possible that the mutant gene may interfere with the hydroxylation of a common hydrocarbon chain from which arise phytol and the carotenols, whereas the carotenes could be formed from this hydrocarbon chain prior to its hydroxylation. Such a common metabolic pathway, in which the formation of carotene hydrocarbons precedes hydroxylated carotenoids, would be in agreement with the suggested order for the synthesis of carotene and carotenols in the yeast, Rhodotorula (7).

The reversion of the mutant seedlings to the normal phenotype has been discussed in a previous paper (13).

Summary

1. It was shown by chromatographic pigment analyses that the seedling mutant, pale-yellow-1, is devoid of chlorophyll a and chlorophyll b when grown in the light. When grown in darkness it contained only 10 % of the amount of protochlorophyll a found in dark-grown normal seedlings. In addition, light-grown mutant seedlings were found to contain less carotenes and carotenols than were present in the normal seedlings. On the other hand, although dark-grown mutant seedlings contained less carotenols than normal seedlings, the same amounts of carotene were found in both mutant and normal seedlings.

2. Eight-day-old, endospermless mutant seedlings contained no protochlorophyll a and virtually no carotenols, while the carotene content was equal to that found in endospermless normal seedlings. This was interpreted to mean that the protochlorophyll a and the carotenols found in the mutant seedling, prior to 8 days after planting, are derived from an endosperm factor, while carotene production is not completely dependent upon this factor. This endosperm factor also contributed to the production of carotenols and carotenes in the normal seedlings.

3. It was found that, 8 days after planting, the mutant block is apparently broken. At this time the light-exposed mutant seedlings begin to accumulate chlorophyll, carotenols and carotenes, whereas the dark-grown mutant seedlings begin to accumulate protochlorophyll a and carotenols, but not the carotenes.

4. The interrelationship of the chlorophylls and the carotenoids were discussed in terms of the action

of the mutant gene. It was suggested that the gene may be operating on a common pathway for the synthesis of the chlorophylls and carotenoids, at a position after the synthesis of carotenes, but before the synthesis of the carotenols and the phytyl portion of the chlorophyll molecule.

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OXIDATIVE PHOSPHORYLATION BY SWEET POTATO MITOCHONDRIA AND ITS INHIBITION BY POLYPHENOLS[1]

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In a previous study (11) it was found that the rate of oxidation of α-ketoglutarate by a mitochondrial preparation from sweet potato roots was considerably enhanced when diphosphothiamine, diphosphopyridine nucleotide, and coenzyme A were added to a basic reaction mixture of adenylate, magnesium, and glucose in a phosphate buffer at pH 7.0. It was of interest, therefore, to examine the phosphorylative capacity of this reconstituted α-ketoglutarate oxidase. The specific purpose of this paper is to report the conditions that have been found for optimum oxidative phosphorylation and the effects of some inhibitors on oxygen and phosphorus uptakes by the particulate fraction of the sweet potato.

Materials and Methods

Key West sweet potatoes (*Ipomoea batatas* Poir) were used in these experiments. The mitochondria were prepared by the methods outlined in another paper (11). The final mitochondrial pellet was suspended in 10 ml of 0.5 M sucrose instead of 6 ml as used for the studies on cofactor requirements. Assay of oxidative activity by Warburg techniques and nitrogen determinations were carried out as already described (11). Inorganic phosphorus was determined by the method of Bernhart and Wreath (2). Each experiment was conducted at least 3 times and the data reported are averages from these experiments.

Results

Effects of Phosphate and Hexokinase on Oxidative Phosphorylation: Rates of oxidation and phosphorylation were studied in increasing concentrations of phosphate in the presence and absence of hexokinase. Phosphorus was determined immediately after the addition of the mitochondria to

[1] Received April 10, 1956.

the reaction mixture (zero-time) by rapidly withdrawing a 1-ml aliquot of the reaction mixture and pipetting into cold 1 M trichloroacetic acid (TCA). A second aliquot was removed and treated similarly after 30 minutes at 25° C. The denatured enzymes were precipitated by centrifugation, and an aliquot of the supernatant was assayed for inorganic phosphorus. The difference in phosphorus content between the "30-minute" and the "zero-time" samples was ascribed to the incorporation of inorganic phosphorus into the organic form.

Phosphate concentrations of 30 micromoles per 3 ml resulted in high oxygen uptake irrespective of the presence of hexokinase (fig 1). With higher concentrations of phosphate little or no further increase in oxygen uptake was noted. The presence of hexokinase caused a significant increase in oxidation at the two lowest phosphate levels. This indicates that

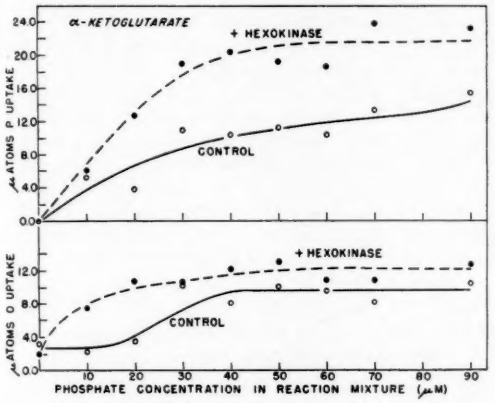

FIG. 1. Oxidative phosphorylation in the α-ketoglutarate system at different levels of phosphate in presence and absence of hexokinase (30-minuate experiment).

Concentration of phosphate is in μM/3 ml.

at low phosphate levels the uptake of oxygen is more closely coupled to the phosphorylating system.

Phosphorus uptake was considerably increased with increase in phosphate concentrations in the range of 0 to 30 micromoles in the medium. However, as the concentration of phosphate was increased from 30 to 90 micromoles, there was little further increase in phosphorus uptake. The actual quantities of phosphorus incorporated into organic form in the 30 to 90 micromole range were approximately 75 to 100 % greater in systems containing hexokinase than in the controls. There was much greater response to hexokinase in phosphorus uptake than in oxygen uptake. This can be explained on the basis of higher phosphorylative efficiency in the presence of hexokinase resulting perhaps in less loss due to phosphatase.

EFFECT OF ETHYLENEDIAMINE TETRAACETIC ACID (EDTA) IN THE PREPARATIVE MEDIUM: It was reported that the chelating agent EDTA stimulated the oxidative activity of mitochondria obtained from

TABLE I

COMPARISON OF OXIDATION AND PHOSPHORYLATION BY SWEET POTATO MITOCHRONDRIA *

PREPARATION MEDIUM	MALONATE	P UPTAKE	O UPTAKE	P/O
	M	*μ atoms*	*μ atoms*	
Sucrose (0.5 M)	0	23.7	11.8	2.0
" + EDTA (0.01 M)	0	36.0	16.8	2.2
Sucrose (0.5 M)	0.01	15.1	5.7	2.7
" + EDTA (0.01 M)	0.01	29.6	8.9	3.3

* The reaction mixture contained: 0.5 M sucrose, 0.02 M PO_4, 0.001 M AMP, 0.02 M glucose, 0.006 M Mg, 0.02 M α-ketoglutarate, 1 mg hexokinase, 3.3×10^{-4} M DPN, 6.6×10^{-5} M DPT, 3.9×10^{-5} M CoA, 0.5 ml mitochondrial suspension containing approximately 0.5 mg protein N; total volume 3 ml; pH 7.1; temp. 25° C; gas phase, air.

Avena seedlings (17) and from broccoli buds (12) and stabilized oxidative phosphorylation mediated by heart muscle sacrosomes (16). Hence the activity of mitochondria prepared in EDTA-sucrose was compared with that in sucrose alone. It can readily be seen (table I) that preparation in EDTA-sucrose considerably increased both oxygen and phosphorus uptake. Phosphorus uptake was increased approximately 50 % in treatments without malonate and approximately 100 % in the malonate treatments. Oxygen uptake was increased approximately 50 % in both treatments.

On the basis of these data all subsequent mitochondrial preparations were homogenized in 0.5 M sucrose + 0.01 M EDTA. In the reaction mixture the phosphate content was 60 micromoles for experiments of 30 to 40 minutes' duration and 90 micromoles for experiments of 1 hour's duration. In all cases 1 mg of hexokinase (Pabst) was also supplied to each vessel.

RATE OF OXIDATIVE PHOSPHORYLATION: The purpose of the next series of experiments was to determine the time course of phosphorus and oxygen uptake during 1 hour's oxidation under optimum con-

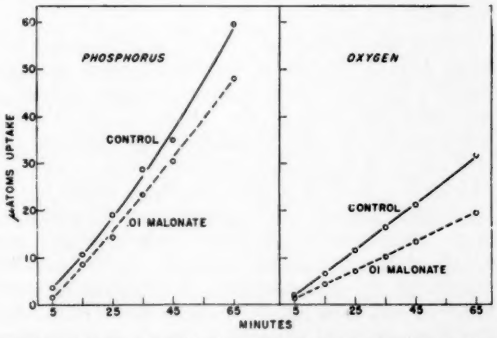

FIG. 2. Rate of oxidation and phosphorylation in the α-ketoglutarate system by sweet potato mitochondria in presence and absence of malonate.

ditions. Presumably if the system were complete the rates of oxidative phosphorylation would be reasonably constant for a considerable period of time. Malonate was added to half of the experimental flasks to limit the oxidation essentially to the single step, α-ketoglutarate to succinate.

From the data in figure 2 it is evident that oxidation and phosphorylation continued at constant rates during a 65-minute period, both in the presence and in the absence of malonate. Malonate inhibited phosphorylation by approximately 15 % and oxygen uptake by approximately 30 %. In the absence of malonate approximately 60 micromoles of phosphorus were incorporated during 1 hour of oxidation. The P/O ratios were approximately 2 in the absence of malonate and approximately 2.5 in its presence.

Effect of Dinitrophenol (DNP): The effect of DNP as a uncoupler of oxidative phosphorylation has been studied by many workers in both animal and

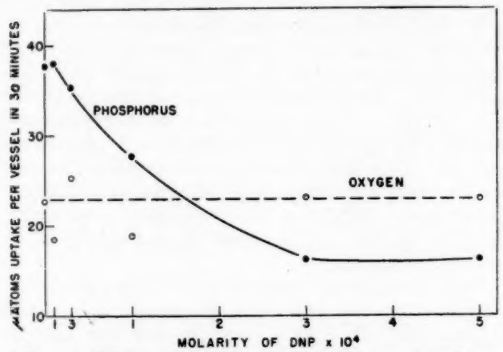

Fig. 3. Effect of dinitrophenol (DNP) on phosphorus and oxygen uptake during 30-minute oxidation of α-ketoglutarate.

plant systems (3, 4, 7, 9). It was of interest to see whether the classical effect of this substance could be demonstrated in the sweet potato mitochondrial system. The effect of various concentrations of DNP on oxygen and phosphorus uptake during the oxidation of α-ketoglutarate in these systems is shown in figure 3.

At all the concentrations tried, which included the range from 1×10^{-5} M to 5×10^{-4} M, there was apparently little consistent change in oxygen uptake as compared with that in the control. A DNP concentration of 1×10^{-4} M inhibited phosphorus uptake about 25 %, while concentrations of 3×10^{-4} M, and 5×10^{-4} M DNP inhibited it about 60 %.

If DNP inhibits all the phosphorylation occurring in the electron transport chain (9), the phosphorus uptake of 17 micro-atoms at 3×10^{-4} M DNP may be considered as substrate-level phosphorylation plus whatever had been lost by phosphatase activity. On this basis the total theoretical phosphorus uptake in the control should be 68+ micro-atoms since the theoretical P/O ratio is 4. However, the experimental value of phosphorus uptake in the control was only 38 micro-atoms with a P/O ratio of 1.7. Apparently part of the electron-transport chain was not phosphorylating. The DNP concentrations effective in inhibiting phosphorylation did not seem to cause concomitant increase in oxygen uptake. On the other hand, when the rates were calculated for different intervals during the experiment, DNP did cause accelerated oxygen uptake, as can be seen in table II. After 20 minutes, the two concentrations that most effectively inhibited phosphorus uptake also markedly accelerated oxygen uptake. Since there was considerable inhibition of oxygen uptake during the first 10 minutes, in the presence of DNP, the true picture of oxygen uptake is distorted if one considers total uptake during a 30-minute experiment. The accelerating effect of DNP on oxidation was more readily seen when the time course of oxygen uptake, in the presence of 3×10^{-4} M DNP, was studied during a 60-minute experiment (fig. 4).

Table II

Effect of DNP on Rate of Oxygen Uptake during 30-Minute Oxidation *

Conc. DNP	Rate of oxygen uptake		
	5–10 Min	10–20 Min	20–30 Min
$M \times 10^{-4}$	*μl/hr (extrapolated)*		
0	444	432	438
5	264	450	546
3	252	456	606
1	336	408	390
0.1	372	348	384

* Reaction mixture same as in table I.

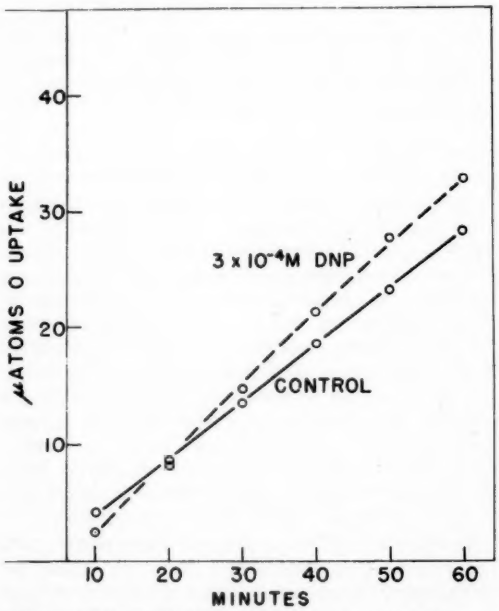

Fig. 4. Effect of DNP on the rate of uptake of oxygen during the oxidation of α-ketoglutarate.

TABLE III

EFFECT OF VARIOUS CONCENTRATIONS OF CATECHOL ON OXIDATION AND PHOSPHORYLATION IN THE α-KETOGLUTARATE SYSTEM

CONC. CATECHOL	O UPTAKE	P UPTAKE	P/O
$M \times 10^{-5}$	*μ atoms*	*μ atoms*	
0*	28.6	49.7	1.7
1	23.0	46.5	2.0
3	27.0	49.0	1.8
10	3.2	0.0	0.0
30	1.6	0.0	0.0
60	0.0	0.0	0.0

* Reaction mixture same as in table I.

EFFECT OF POLYPHENOLS ON OXIDATIVE PHOSPHORYLATION DURING THE OXIDATION OF α-KETOGLUTARATE: In view of the pronounced effect of DNP in uncoupling oxidation from phosphorylation, it had been postulated (13) that natural substances in the cell may function similarly as uncouplers, especially during senescence. Akazawa and Uritani (1) reported that chlorogenic acid, a natural polyphenolic substance in sweet potatoes (15), uncoupled oxidation from phosphorylation during the oxidation of a Krebs cycle intermediate. We decided, therefore, to use catechol, a simple polyphenolic substance, to test the effect of polyphenols on the oxidation of α-ketoglutarate by the sweet potato mitochondria in the complete system.

Concentrations of catechol up to 3×10^{-5} M had no effect on oxidation or phosphorylation (table III). However, concentrations of 10^{-4} M and higher almost completely inhibited both oxidation and phosphorylation. Since catechol is very easily oxidized to the quinone form it seemed plausible to assume that oxidation of catechol occurred and was related to the inhibition of oxidative phosphorylation in these systems. The mitochondria exhibited some polyphenolase activity indicating that catechol could be oxidized by the mitochondria.

When ascorbic acid, which can keep the polyphenol in the reduced state, was added to the complete α-ketoglutarate system containing catechol, the inhibitory effect was prevented (table IV). Substitution of chlorogenic acid for catechol in these experiments gave identical results (table IV). From these data it was concluded that the oxidized polyphenols (quinones) or their polymerization products strongly inhibited oxidative phosphorylation in the α-ketoglutarate system and that the reduced form of the polyphenol was innocuous.

TABLE IV

EFFECT OF POLYPHENOLS AND POLYPHENOLS PLUS ASCORBIC ACID ON OXIDATION AND PHOSPHORYLATION IN THE α-KETOGLUTARATE SYSTEM *

FLASK CONTENT	O UPTAKE	P UPTAKE	P/O
	μ atoms	*μ atoms*	
Control	15.1	27.0	1.8
" + ascorbic acid (3×10^{-4} M)	10.9	22.6	2.1
" + catechol (3×10^{-4} M)	1.1	2.6	...
" + catechol + ascorbic acid	14.1	31.7	2.2
" + chlorogenic acid (3×10^{-4} M)	2.2	0.0	...
" + chlorogenic and ascorbic acids	15.8	27.6	1.7

* Duration of the experiment 30 min. Reaction mixture of the control same as in table I.

It was still not clear whether the oxidation of the polyphenols was spontaneous or enzymatic. An attempt was made to study this by addition of diethyldithiocarbamate (DIECA), a chelating agent which inhibits copper enzymes (8), to the flasks containing catechol and the α-ketoglutarate-oxidase system. In the presence of DIECA there was no inhibition of the α-ketoglutarate system by catechol (table V). This can be interpreted to mean that the inhibition of α-ketoglutarate oxidation by catechol is dependent on the oxidation of catechol by polyphenolase or copper. There was probably no free copper present, since the mitochondrial preparations were made in the presence of EDTA and all solutions were prepared with doubly glass-distilled water. Therefore, it seems that catechol was oxidized by polyphenolase. However, the possibility of oxidation by a metal-protein-bound moiety exists.

TABLE V

EFFECT OF DIECA ON CATECHOL INHIBITION OF THE α-KETOGLUTARATE SYSTEM

FLASK CONTENT	Q_{O_2} (N)
Control*	564
" + DIECA (6×10^{-5} M)	627
" + catechol (3×10^{-4} M)	0
" + catechol + DIECA	520

* Reaction mixture same as in table I.

DISCUSSION

Attempts have been made by plant biochemists to obtain high efficiencies of phosphorylation similar to those reported for animal tissue (7, 9). With mung bean (4) the P/O ratio was found to be 1, while for the avocado (3) and the cauliflower (10) occasional values exceeding 2 were reported. Recently a P/O of 3.0 was obtained for lupine mitochondria (5). This study with sweet potato indicates that consistently good rates of phosphorus uptake can be achieved with a reconstituted mitochondrial preparation. Although, in the one-step oxidation from α-ketoglutarate to succinate, P/O ratios approaching 3 were obtained, the evidence from DNP inhibition indicates that nearly half of the phosphorylation takes place at the substrate level. This implies that the electron-transport chain is not phosphorylating at maximum capacity.

The data on inhibition of oxidative phosphorylation by polyphenols and its prevention by ascorbic acid can be used as a partial explanation of the course

of senescence in plant tissue. In the normal active cell the oxidation of polyphenols must be coupled to a system that immediately reduces the quinone formed. Ascorbic acid or similar reducing substances may function in this capacity. Darkening due to polymerization of quinones is, therefore, not observed in young normal cells, though the cells may contain polyphenols and polyphenolases. However, if ascorbic acid, or other specific reducing systems, is lost during the aging process, quinones would gradually build up in the cells. The presence of quinones, which rapidly polymerize, results in inhibition of both oxidation and phosphorylation of the mitochondrial system and eventually causes death.

Ezell and Wilcox (6) presented data showing that about 50 % of the ascorbic acid in sweet potatoes was lost in storage. Other data indicate that ascorbic acid declines in plant tissues with age (14). The darkening generally observed when cells are injured and exposed to the atmosphere may be due to the abundance of oxygen which causes the oxidation of the quinone-reducing systems. This consequently allows polymerization of quinones. In general darkening is characteristic of aged tissue. The build-up of quinones during the aging process is probably gradual and does not become detrimental until a concentration of approximately 10^{-4} M is reached. The scheme suggested proposes a possible role of the polyphenol-ascorbic acid systems in senescence which merits further study.

Summary

A mitochondrial system from sweet potatoes, prepared in EDTA-sucrose, carried out oxidative phosphorylation at very good rates during oxidation of α-ketoglutarate. The oxidation and phosphorylation proceeded at constant rates for at least 1 hour. In this time approximately 60 micro-atoms of phosphorus were taken up and a P/O ratio of approximately 2 was obtained. In the presence of malonate the P/O ratios approached 3.

DNP uncoupled oxidation from phosphorylation at concentrations of 1×10^{-4} M, 3×10^{-4} M, and 5×10^{-4} M. Concentrations lower than 1×10^{-4} M had little effect.

The polyphenols, catechol and chlorogenic acid, in concentrations of 3×10^{-4} M almost completely inhibited phosphorylation and oxidation during the dissimilation of α-ketoglutarate by the mitochondrial system. This inhibition was prevented by ascorbic acid or DIECA, suggesting that the quinone form is the inhibitor.

A scheme which attributes senescence in plant cells to interaction of the polyphenol-ascorbic acid systems was proposed.

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COFACTOR REQUIREMENTS FOR OXIDATION OF ALPHA-KETO ACIDS BY SWEET POTATO MITOCHONDRIA[1]

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In recent years evidence that suggests the operation of the Krebs cycle in plant tissues has accumulated. Studies by Millerd et al (10), Laties (8), Brummond and Burris (4), Biale and Young (3), and Smillie (14), have demonstrated that mitochondria, isolated by differential centrifugation of tissue homogenates, can carry out oxidation with concomitant phosphorylation of Krebs cycle acids. Davies (5), and Abramsky and Biale (1) have shown by chromatographic techniques that the oxidations of Krebs cycle acids are, in fact, mediated through the Krebs cycle and that pyruvate enters the cycle. However, active mitochondria have been isolated from plants of few species. Most of the tissues used for these studies, except those of Biale and Young (3) and Smillie (14), were either juvenile or etiolated. There is, therefore, a need to extend the knowledge of mitochondrial oxidations to many more plant species and to different tissues. The objective of this study was to isolate and characterize mitochondria from sweet potato roots (*Ipomoea batatas* Poir.). It was hoped that work with a different species and a different tissue might throw some additional light on mitochondrial activity in plant cells.

In most metabolic studies with cytoplasmic particles from plants the rates of pyruvate oxidation were considerably lower than those of the other acids. The cofactors used for activating pyruvate oxidase were virtually the same as those for malate, citrate, etc. The requirement for a primer acid, adenylate, and magnesium was established in the early investigations. It seemed desirable, therefore, to explore more fully the relation of some of the less commonly used cofactors to the oxidation of keto acids. This paper reports the results of such studies.

MATERIALS AND METHODS

The sweet potatoes used in these experiments were of the Key West variety. Particulate suspensions were prepared by the differential centrifugation technique as outlined by Biale and Young (3). The roots were chilled at 0° C for 1 hour, peeled, grated, and 200 gm of grated material was homogenized with 300 ml of 0.5 M sucrose in a Waring blendor. Blending was carried out for 2 minutes at 47 volts. The homogenate was centrifuged at 1000 × g for 5 minutes to precipitate starch, whole cells, cell walls, and other large cellular fractions. The supernatant was centrifuged at 14,000 × g for 15 minutes; the precipitated pellet was washed in 20 ml of 0.5 M sucrose and again centrifuged at 14,000 × g for 15 minutes. The resulting precipitate was suspended in 6 ml of 0.5 M sucrose and used as the particulate fraction or the "mitochondrial" preparation. The preparations contained approximately 0.5 mg N per 0.5 ml of suspension. Up to this point all manipulations were carried out at 0° C.

The particles were assayed for activity by standard Warburg manometric techniques at 25° C with air as the gas phase. The volume of the reaction mixture was 3 ml and the solutions added to the vessels are given in the tables and figures or in the appropriate paragraph under "Results."

Nitrogen determinations on the isolated mitochondria were made by Nesslerization of an aliquot of the mitochondrial suspension according to the method of Thompson and Morrison (17) as modified by Biale and Young (3).

In every case the values reported in the tables and figures are averages from at least 3 experiments. Abbreviations used are as follows: $Q_{O_2}(N)$, μl O_2 uptake per mg nitrogen per hour; AMP, adenosine monophosphate; DPN; diphosphopyridine nucleotide; CoA, coenzyme A; and DPT, diphosphothiamine.

RESULTS

OXIDATION OF KREBS CYCLE INTERMEDIATES: The rates of oxidation of several intermediates of the Krebs cycle by the mitochondrial suspension of sweet potato are shown in table I. The oxidation values in table I compare favorably with those reported for other plant tissues for the oxidation of citrate, α-ketoglutarate, and succinate (3, 5, 10). However, it appeared that the ability to oxidize pyruvate was almost totally lacking in sweet potato mitochondria. It, therefore, seemed desirable to study the pyruvate oxidase system in the sweet potato.

COFACTOR REQUIREMENTS FOR PYRUVATE OXIDATION: The general scheme for cofactor cycling in the

TABLE I

OXIDATION OF KREBS CYCLE ACIDS BY SWEET POTATO MITOCHONDRIA *

SUBSTRATE	Q_{O_2} (N)
None	0
Pyruvate **	35
Citrate	216
α-Ketoglutarate	179
Succinate	432

* Reaction mixture consisted of 0.5 M sucrose, 0.001 M AMP, 0.02 M glucose, 0.006 M Mg, 0.01 M P_{O_4}, 0.02 M substrate, and 0.5 ml mitochondrial preparation containing approximately 0.5 mg N, pH 7.1.

** The concentration of malate as a primer was 0.0005 M.

[1] Received April 10, 1956.

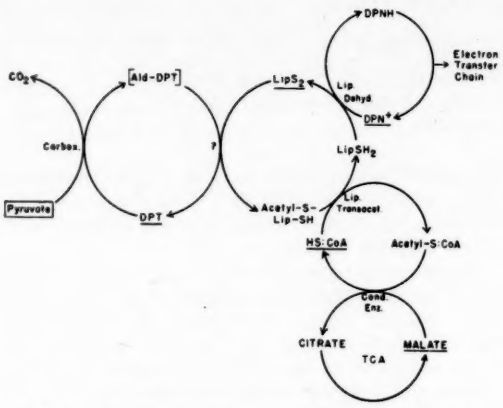

FIG. 1. Scheme for cofactor requirements and cycling in the oxidation of pyruvate [after Gunsalus (7)]. Abbreviations are as follows: Carbox, carboxylase; Ald-DPT, aldehyde-diphosphothiamine complex; Lip-S_2, oxidized lipoic acid; Acetyl-S-Lip-SH, acetyl lipoic complex; Lip-SH_2, reduced lipoic acid; Lip. Dehyd., lipoic dehydrogenase; Lip. Transacet., lipoic transacetylase; HS:CoA, reduced CoA; acetyl-S:CoA, acetyl CoA; Cond. Enz., condensing enzyme. TCA-tricarboxylic acid cycle. The underlined cofactors were selected for this study.

oxidation of pyruvate, as worked out in soluble systems from animal tissues and microorganisms, is presented in figure 1. This diagram, adapted from Gunsalus (7), indicates that DPT, lipoic acid, DPN, CoA, and malate are all involved in pyruvate oxidation.

TABLE II

EFFECTS OF COFACTORS INDIVIDUALLY, IN PAIRS, AND COLLECTIVELY ON OXIDATION OF PYRUVATE BY SWEET POTATO MITOCHONDRIA

COFACTOR* ADDED TO THE BASIC MIXTURE**	Q_{O_2} (N)
None	20
DPN	90
DPT	153
CoA	16
Lipoic acid	12
None	30
DPT	153
DPT + DPN	232
DPT + CoA	119
DPT + lipoic acid	140
None	20
DPN	82
DPN + DPT	334
DPN, DPT + CoA	302
DPN, DPT, CoA + lipoic acid	278

* Concentrations of the cofactors: DPN 3.3×10^{-4} M; DPN 6.6×10^{-5} M, CoA 3.9×10^{-5} M and lipoic acid 3.3×10^{-5} M.

** Content of the basic reaction mixture is given in table I.

On the assumption that these cofactors are similarly concerned in the oxidations by plant mitochondria, it seemed possible that one or more cofactors may have been lost from the mitochondria during their isolation. To test this, systematic additions of cofactors were made to the basic reaction mixture containing the mitochondria and pyruvate as substrate. The basic reaction mixture contained 0.5 M sucrose, 0.01 M PO_4 pH 7.1, 0.001 M AMP, 0.02 M glucose, 0.006 M Mg, 0.0005 M malate as a "sparker," 0.02 M pyruvate, and 0.5 ml of the particulate suspension. To this basic reaction mixture were added singly, doubly, and collectively 3.3×10^{-4} M DPN, 6.6×10^{-5} M DPT, 3.9×10^{-5} M CoA and 3.3×10^{-5} M lipoic acid (kindly supplied by Dr. L. J. Reed of the University of Texas). The results of these studies are shown in table II.

These data (table II) show that good oxidation of pyruvate was obtained only when DPT and DPN were added to the basic reaction mixture. While DPT or DPN alone increased activity markedly, the simultaneous addition of these two cofactors caused an additive increase. The cofactor DPT caused the greater stimulatory effect. On the other hand, neither CoA nor lipoic acid caused any stimulation of activity. This indicated that the addition of these cofactors was not required for the oxidation of pyruvate by isolated sweet potato mitochondria.

TABLE III

MAGNESIUM REQUIREMENT FOR PYRUVATE OXIDATION BY SWEET POTATO MITOCHONDRIA*

Mg CONC. $\times 10^{-3}$ M	Q_{O_2} (N)
0	129
0.1	133
1	245
3	247
6	213

* All cofactors added in amounts indicated in table II.

In all the experiments just reported a magnesium concentration of 0.006 M was used as a basic cofactor. However, Young and Biale (18) reported that magnesium concentrations of 0.006 M strongly inhibited pyruvate oxidation by avocado mitochondria. The magnesium requirement for pyruvate oxidation in sweet potato was therefore studied (table III). According to these data magnesium concentrations greater than 10^{-4} M are required in this system for optimal activity. In subsequent experiments a concentration of 10^{-3} M magnesium was added to the cofactor mixture used to mediate pyruvate oxidation.

The importance of adding a primer such as malate in the oxidation of pyruvate has been noted by Millerd (11), Brummond and Burris (4), Tager (16) and others. The purpose of a primer acid is to furnish a condensing partner for acetyl-CoA so that the two-carbon fragment resulting from the oxidative decarboxylation of pyruvate can be introduced into the

TABLE IV

PRIMER ACID REQUIREMENT FOR PYRUVATE OXIDATION BY SWEET POTATO MITOCHONDRIA

COFACTOR ADDITION	MALATE CONC. $\times 10^{-4}$ M	Q_{O_2} (N)
Basic *	0	8
Basic + DPN + DPT	0	12
Basic	5	20
Basic + DPN + DPT	5	302

* For addenda see tables I and II.

Krebs cycle. This reaction is mediated through the condensing exzyme (fig 1), which is presumed to be present in the mitochondria.

Table IV shows that in these systems the priming effect of malate was extremely critical. In the absence of malate there was no oxidation of pyruvate, even though DPN and DPT were present. Conversely, in the presence of the complete basic reaction mixture including malate, there was virtually no oxidation when DPT and DPN were absent.

OXIDATION OF α-KETOGLUTARATE: After obtaining such a striking response to DPT and DPN by the pyruvate oxidase system of sweet potato mitochondria, the importance of these cofactors in the α-ketoglutarate oxidase system was studied. Table V shows the results obtained. The basic reaction mixture contained the same cofactors as for pyruvate oxidation (including 0.006 M magnesium) except that no malate primer was used. It is evident from the data that the collective addition of DPT, DPN, and CoA to the basic reaction mixture considerably increased the oxidation rate of α-ketoglutarate by sweet potato mitochondria. The addition of CoA alone or in combination with DPT caused little increase of the oxidative rate. However, when CoA was added to DPN and DPT there was a marked increase in oxidation rate, indicating a synergistic effect. In this respect the mitochondria reacted differently to the addition of CoA when α-ketoglutarate was oxidized than when pyruvate was oxidized. This may be explained by the considerably higher Q_{O_2}(N) obtained with α-ketoglutarate (approximately 500) than with pyruvate (approximately 300). CoA presumably became limiting at the higher oxidation levels.

TABLE V

EFFECTS OF COFACTORS INDIVIDUALLY, IN PAIRS, AND COLLECTIVELY ON OXIDATION OF α-KETOGLUTARATE BY SWEET POTATO MITOCHONDRIA

COFACTOR ADDED TO THE BASIC MIXTURE *	Q_{O_2} (N)
None	167
DPN	218
DPT	316
CoA	188
Lipoic acid	174
None	178
DPT	316
DPT + DPN	384
DPT + CoA	277
DPT + lipoic acid	249
None	162
DPN	224
DPN + DPT	419
DPN, DPT + CoA	575
DPN, DPT, CoA + lipoic acid	575

* For addenda see tables I and II.

TABLE VI

EFFECTS OF ARSENITE ON OXIDATION OF α-KETO ACIDS *

SUBSTRATE	ARSENITE CONC. $\times 10^{-4}$ M	Q_{O_2} (N)
Pyruvate	0	228
"	5	17
α Ketoglutarate	0	336
"	5	21

* All cofactors added to the reaction mixture (see tables I and II).

ARSENITE INHIBITION: With both α-keto acids there was no response to the addition of lipoic acid. Since arsenite was reported (13) to be a fairly specific inhibitor of enzymes containing active disulfhydryl groups such as lipoic acid, the effect of arsenite in the reaction mixture was studied (table VI). Arsenite completely inhibited the oxidation of pyruvate and α-ketoglutarate, indicating that a lipoic acid like substance is probably present in the sweet potato mitochondria.

CHROMATOGRAPHIC EVIDENCE FOR THE OPERATION OF THE KREBS CYCLE IN THE SWEET POTATO: In order to obtain direct evidence for the formation of Krebs cycle intermediates chromatographic techniques were used to follow the products of the oxidative reactions. The reaction mixture in the Warburg flasks were chromatographed at zero time (immediately after addition of enzyme) and after 120 minutes. After adjustment to pH 5.2, the enzymes were

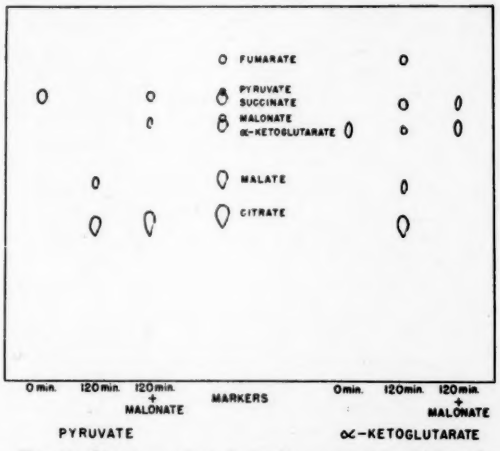

FIG. 2. Diagram of a chromatogram of reaction mixtures containing all cofactors at zero time and after 120 minutes of oxidation.

denatured by heating for 2 minutes, and the mixture was cleared by centrifugation. The clear solution was chromatographed with butanol–5 M formic as the developer and bromphenol blue as the indicator, according to the method of Lugg and Overell (9).

Figure 2 shows one of the chromatograms obtained. At zero time only a spot for the substrate, either pyruvate or α-ketoglutarate, was visible. However, after 120 minutes of reaction in the Warburg vessel the spot for the substrate diminished and other spots, which were identified as intermediates of the Krebs cycle, appeared. When pyruvate was the substrate, spots for citrate and malate appeared. When α-ketoglutarate was the substrate, spots for citrate, malate, succinate and fumarate appeared. In the presence of 0.01 M malonate only succinate was visible on the chromatogram. These data strongly suggest that the oxidations studied were mediated through the Krebs cycle.

Discussion

All the cofactors necessary for oxidation of pyruvate, as outlined in figure 1, are accounted for in these experiments except CoA. However, evidence from the α-ketoglutarate system implies that sufficient CoA is present in the mitochondria for the level of pyruvate oxidation obtained. The cofactor requirements for α-ketoglutarate oxidation are assumed to be essentially the same as for pyruvate except that no priming acid is needed. A requirement for CoA in the α-ketoglutarate system does not become apparent until DPT and DPN are added and the $Q_{O_2}(N)$ is approximately 400. Since the level of pyruvate oxidation is considerably lower with DPT and DPN there is no response to CoA addition. Lipoic acid is presumed to be present in the mitochondria from the data on arsenite inhibition.

Akazawa and Uritani (2) isolated mitochondria from sweet potatoes but did not obtain much activity with any of the Krebs cycle acids as substrates. The data reported herein indicate that an active mitochondrial system can be isolated from sweet potatoes. However, these preparations could not oxidize pyruvate at all and oxidized α-ketoglutarate at a fairly good rate. The addition of suitable cofactors to the reaction mixtures strikingly increased oxidation of both substrates. The assumption is that the basic structure of the isolated mitochondria necessary for these oxidations was intact, since they oxidized the substrate at very good rates after the addition of suitable cofactors.

The absence of cofactors from the isolated mitochondria can be explained in one of two ways. It can be assumed that the isolating procedure removes or destroys the cofactors from the mitochondria by solubilization, by extraction, or by other means, without altering their basic structure. The function of the mitochondria, therefore, appears reversible with respect to these cofactors. It is possible that the enzymes in question are either on the surface of the mitochondria or in the mitochondrial membrane or are in some way made easily accessible to added cofactors. The electron microscope photographs of Palade (12) and Sjostrand (15) show mitochondrial membranes that may well contain enzymes. Apparently lipoic acid is rather firmly attached and remains within the mitochondrial framework after isolation. It is assumed that in vivo the cofactors are directly associated with their enzymes in the mitochondria. Another possible explanation is that the cofactors are not present in the mitochondria, in vivo, but are in the cytoplasmic environment surrounding the mitochondria in the cell. In this arrangement there must be rapid and complete interaction between the enzymes located in the mitochondria and their coenzymes in the surrounding cytoplasmic medium. This concept is not in agreement with Green's (6) picture of the mitochondria as independent integrated enzyme systems (the cyclophorase) containing all the necessary cofactors within their structure.

Summary

Mitochondria isolated from sweet potatoes oxidized most Krebs cycle acids in the presence of AMP, Mg, glucose, and phosphate, but could not oxidize pyruvate. The addition of the cofactors DPT and DPN to the reaction mixture brought about high rates of pyruvate oxidation. The oxidation of α-ketoglutarate was increased more than 200 % by addition of DPT, DPN, and CoA. Exogenous lipoic acid had no effect with pyruvate or α-ketoglutarate as substrate. However, arsenite completely inhibited the oxidation of both α-keto acids indicating the possible presence of lipoic acid in the mitochondria. Chromatographic evidence suggests that the oxidation of the α-keto acids by these mitochondria is mediated through the Krebs cycle.

Since the preparation of these manuscripts the articles by Beevers, H., and Walker, D. A. (Biochem. Jour. 62: 114–119. 1956) and by Walker, D. A., and Beevers, H. (Biochem. Jour. 62: 120–127. 1956) appeared. They used the same cofactors and arrived at substantially the same results with castor bean seedlings as we did with the sweet potato.

The authors wish to express their appreciation for the technical assistance of Harold Neimark.

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FLORAL INITIATION IN CESTRUM NOCTURNUM, A LONG-SHORT DAY PLANT. II. A 24-HOUR VERSUS A 16-HOUR PHOTOPERIOD FOR LONG DAY INDUCTION[1]

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Recent experiments have shown that *Cestrum nocturnum* is a long-short day plant, requiring first long day and then short day induction (5). The purpose of this paper is to make some comparisons between long day induction in Cestrum and in simple long day plants.

METHODS, RESULTS, AND DISCUSSION

The experiments were performed in the Earhart Laboratory (6) using the 26° C greenhouse and 20° C temperature controlled rooms (for both artificial light and darkness). The light intensity was generally in excess of 1500 ft-c in the greenhouse and approximately 700 ft-c at plant tops in the artificial light rooms in which the light was supplied by warm, white fluorescent tubes supplemented with incandescent bulbs.

All of the plants used were grown from seed and were more than 3 months old at the beginning of long day induction. The criteria for the selection of plants and quantitative measurement of floral initiation were the same as those described previously (5). The errors quoted for the number of nodes flowering per plant are the standard errors of the means; the "t" test (3) was applied assuming that the samples were taken from normal distributions.

Two groups of plants were exposed to 12 or 18 long days with a 16-hour photoperiod (8 hours in the greenhouse plus 8 hours of artificial light and 8 hours of darkness). Two other lots received 12 or 18 long days with a 24-hour photoperiod (8 hours in the greenhouse plus 16 hours of artificial light). At the end of the respective long day treatments the plants were transferred to short day conditions (8 hours in the greenhouse plus 16 hours of darkness) for 7 days. The results of the experiment, recorded in table I, show that for 12 days a 16-hour photoperiod was greatly superior to a 24-hour photoperiod for long day induction. As the number of long days was increased the difference between the two treatments decreased. This experiment was repeated twice with essentially the same results. Apparently in the 8 hours of darkness between the end of one 16-hour photocycle and the beginning of another, a reaction(s) may be activated by light which reduces the effectiveness of long day induction.

It has been reported for long day plants that continuous light (24-hour photoperiod) is the most

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TABLE I

TWENTY-FOUR-HR VS 16-HR PHOTOPERIOD FOR LONG DAY INDUCTION

PP †	No. of LD †	% Fl †	% Term Fl †	Nodes Fl/Pl †	No. of plants
24	12	100	0	6 ± 1 *	8
16	12	100	71	12 ± 2 *	7
24	18	100	75	17 ± 2 **	8
16	18	100	100	20 ± 1 **	8

* Difference significant at 5 % level.

** Difference not significant at 5 % level.

† PP, photoperiod; LD, long days; % Fl, percent of plants with flowers; % Term Fl, percent of plants with terminal flowers; Nodes Fl/Pl, number of nodes flowering per plant.

favorable condition for floral initiation (1, 2). Since this is not the case for Cestrum there may be a basic difference between long day induction in it and in simple long day plants. On the other hand perhaps this behavior is due to the requirement for subsequent short day induction. Harder and Bünsow (4) reported that floral initiation in *Kalanchoë Blossfeldiana*, a short day plant, was inhibited if plants were exposed to long days prior to short day induction; as the length of the photoperiod of the preceding long days was increased the effectiveness of short day induction was decreased. The authors suggested that an inhibitory substance was formed in the long photoperiods. In a previous paper concerning Cestrum (5) it was shown that the effect of long day induction was confined to the treated leaves and it was concluded that the hypothetical product of long day induction was not translocated from the leaves. In addition evidence was presented showing that this product was a precursor of the floral stimulus. If along with the floral stimulus precursor an inhibiting substance were formed, as suggested for *K. Blossfeldiana*, perhaps the explanation for the reduced effectiveness of a 24-hour photoperiod is that the ratio of inhibitor to precursor is highest in this day-length. The precursor accumulates in the leaves and, if the inhibitor were dissipated by translocation or destruction, the ratio of inhibitor to precursor would decrease with prolonged long day induction. This would explain why the difference between a 24- and 16-hour photoperiod decreased as the number of long days increased.

SUMMARY

It has been shown that a 16-hour photoperiod is superior to a 24-hour photoperiod for long day induction in *Cestrum nocturnum*, a long-short day plant. It was suggested that, along with a promoting substance, an inhibitor is formed during the photoperiod.

The author would like to acknowledge the advice and encouragement of Dr. F. W. Went and the help of the staff of the Earhart Laboratory throughout the course of this investigation.

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FLORAL INITIATION IN CESTRUM NOCTURNUM, A LONG-SHORT DAY PLANT. III. THE EFFECT OF TEMPERATURE UPON LONG DAY AND SHORT DAY INDUCTION[1]

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Previous reports of temperature effects upon photoperiodic induction were concerned with long day plants (LDP) and short day plants (SDP) (4). Recently a new group of photoperiodically sensitive plants was discovered, the long-short day plants, which require long day (LD) followed by short day (SD) induction for floral initiation (6). The purpose of this paper is to present the results of investigations on the effect of temperature upon LD and SD induction in *Cestrum nocturnum*, a long-short day plant (7).

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METHODS AND MATERIALS

All experiments were performed in the Earhart Laboratory (9). The temperatures in the greenhouses and artificial light rooms were controlled to

within ± 1° C (all temperatures are in degrees Centigrade). Twice daily the plants were exposed to a temperature of approximately 23° for periods of ½ hour each, the time required to transfer them from one set of temperature and light conditions to another. The light intensity in the greenhouses was generally in excess of 1500 ft-c and approximately 700 ft-c in the artificial light rooms where the light was supplied by warm, white fluorescent tubes supplemented with incandescent bulbs. Unless otherwise noted LD refers to a 16-hour photoperiod (8 hours in the 26° greenhouse plus 8 hours of artificial light and an 8-hour nyctoperiod at 20°) and SD refers to a 16-hour nyctoperiod (8 hours in the 26° greenhouse plus a 16-hour nyctoperiod at 20°).

The methods employed in growing Cestrum plants and evaluating quantitative differences among treatments were the same as those described previously (7). The effect of temperature upon vegetative growth was estimated by recording the number of leaves unfolded from the terminal apex during LD induction. All figures in the tables have been rounded off to the nearest whole number.

Results and Discussion

Long Day Induction, *Phototemperature:* In a series of experiments Cestrum plants were exposed to a 16-hour photoperiod (16 hours in artificial light rooms at various temperatures plus an 8-hour nyctoperiod at 20°) for 18 days. At the end of this treatment the plants were transferred to SD conditions for 7 days. The results of duplicate experiments were averaged together and recorded in table I. These

Table I

The Effect of Temperature upon Long Day Induction

Temp.	% Fl**	% Term Fl	Nodes Fl/Pl*	No. of leaves unfolded	No. of plants
		A. Phototemperature			
30	100	100	10 ± 1	7	5
26	100	50	14 ± 2	9	16
23	100	78	18 ± 2	9	13
20	100	57	15 ± 2	11	14
17	100	29	15 ± 2	11	14
14	75	0	3 ± 1	9	8
10	34	0	2 ± 1	4	14
7	0	0	0	4	13
		B. Nyctotemperature			
26	83	67	11 ± 4	12	6
23	100	67	14 ± 3	10	6
20	100	67	14 ± 1	9	6
17	100	50	13 ± 3	10	6
14	100	67	16 ± 3	10	6
10	100	67	15 ± 4	10	6
4	100	50	14 ± 3	4	6

* The errors quoted for the Nodes Fl/Pl represent the standard errors of the means.

** Abbreviations used: % Fl, percent flowering, % Term Fl, percent terminal flowering; Nodes Fl/Pl, number of flowering nodes per plant.

experiments showed clearly that the phototemperature of LD induction had a great effect upon floral initiation. In a previous work (7) it was shown that the effect of LD induction was confined to the leaves and floral initiation did not occur until after SD induction was complete. Therefore, temperature treatments during LD induction should have affected only inductive reactions and processes such as photosynthesis, respiration, cell elongation and division, but not translocation of the floral stimulus or initiation of the buds. In the conditions in which vegetative development was affected concomitantly, it cannot be said that the temperature effects were specific for photoperiodic processes. In a few cases, however, a specific temperature effect upon LD induction was evident. For example, there was no difference in the number of leaves unfolded between the 10 and 7° groups and the absence of floral initiation at the lower temperature probably reflected the minimum phototemperature for LD induction. A comparison of the vegetative growth and floral initiation data between the 14° group on the one hand and the 30, 26, and 23° groups on the other shows that the effectiveness of LD induction was greatly reduced at the lower phototemperature whereas vegetative development was not. Since both the percent of plants with terminal flowers and the number of flowering nodes decreased at 26° (as compared with the values for the 23° group) and the number of flowering nodes also at 30°, it seems unlikely that the increase in percent terminal flowering at 30° was due to the increased effectiveness of LD induction at this high temperature (in fact this was the only case in more than 100 experiments in which the trends for percent terminal flowering and number of flowering nodes did not agree). Perhaps the 30° treatment during LD induction reduced the response of the lower nodes to floral initiation thereby insuring an excess of stimulus for translocation to the terminal apex and a high value for percent terminal flowering. Whatever the reason it is probable that the optimum phototemperature for LD induction is near 23°.

Nyctotemperature: In a single experiment a number of groups of plants were exposed to 18 LD (16 hours of artificial light at 26° plus an 8-hour nyctoperiod at various temperatures) and then transferred to SD conditions for 7 days. The results of the experiment, recorded in table I, show that the nyctotemperature had very little effect upon LD induction. This was true even with a 4° nyctotemperature in which vegetative growth was severely inhibited. Perhaps the reason for the temperature insensitivity of the nyctoperiod is that the length of the induction period obscured the differences among the various treatments, as in the case in which the differences between a 24- and 16-hour photoperiod for LD induction were greatly reduced after 18 LD (8). This possibility should be checked with experiments in which the number of LD is decreased. Since light given during this 8-hour nyctoperiod inhibited LD induction (8), it will be particularly

interesting to learn whether these 8 hours are truly temperature insensitive, with and without light.

Comparisons between experiments with Cestrum and those with LDP are difficult owing to the different experimental conditions and criteria for evaluation of floral initiation. In Hyoscyamus, with a 10-hour photoperiod, Lang and Melchers (2) found that the number of days to floral initiation was reduced by lowering the phototemperature from 29 to 18° (nyctotemperature constant at 27.5°). Experiments with differential photo- and nyctotemperatures were not extended, but, with the same temperature throughout, the critical daylength was reduced by lowering the temperature within the range of 28.5 to 15.5°. The impression from these experiments was that lowering the temperature increased the effectiveness of LD induction. Liverman and Lang (3) found for Silene, with a 14-hour photoperiod, that varying the phototemperature between 10 and 23° had no effect upon the number of days to floral initiation although at 30° there was a significant inhibition. Within this same range the nyctotemperature had a great effect upon LD induction; with a 14-hour photoperiod, increasing the nyctotemperature from 10 to 30° (phototemperature constant at 23°) greatly promoted floral initiation. There is no doubt from these experiments that in Silene the nyctotemperature plays the most important role in LD induction. Cestrum is completely different since, with a 16-hour photoperiod, the phototemperature had a far greater effect than the nyctotemperature upon LD induction. Perhaps with a shorter photoperiod the effect of the photo- and nyctotemperature upon LD induction in Cestrum would approach that in Silene although there is reason to believe that the two plants should react differently to temperature during induction. The floral stimulus in Cestrum is not synthesised during LD induction but appears only after subsequent SD induction (7), whereas in Silene the floral stimulus is synthesized as a result of LD induction alone (3). If the floral stimulus is identical in the two plants and LD induction involves the synthesis of similar substances, the reaction which leads to the final synthesis of the stimulus, and which in Cestrum requires SD, must occur in Silene, too, but without photoperiodic control. Hence in Silene temperature treatments during LD induction may be affecting at least one additional process not affected in Cestrum.

Borthwick, Hendricks, and Parker (1) have suggested, on the basis of light interruption experiments, that floral initiation in both SDP and LDP may be controlled by the conversion of a pigment from a red to far-red absorbing form. The synthesis of the floral stimulus (or precursor) in SDP occurs while the pigment is in the red absorbing form and in LDP when predominantly in the far-red state. The transformation of the pigment from one form to another accounts for the photosensitized reactions; however, it has been shown recently by Downs for Xanthium, a SDP, that the photoreaction is coupled to a temperature dependent reaction (see discussion and reference in review by Liverman (4)). Presumably Downs's observations would apply to LDP as well. In LDP then the phototemperatures should have a great effect upon LD induction since it is during the photoperiod that the pigment is in the far-red absorbing form and the thermochemical reactions involved in the synthesis of the floral stimulus (or precursor as in the case of Cestrum) may occur. This is not to say that according to the "pigment" theory the dark period should be completely temperature insensitive; however, presumably greater weight would be given to the phototemperature. The facts for Cestrum are in accord with this reasoning, whereas the apparent temperature insensitivity of the photoperiod in Silene suggests that some additional explanation may be necessary.

Short Day Induction, *Phototemperature:* In a single experiment Cestrum plants were exposed to 20 LD followed by 3 SD (8-hour photoperiod in artificial light at various temperatures plus a 16-hour nyctoperiod at 20°). After SD induction the plants were returned to LD conditions for 4 days. The results of the experiment are recorded in table II. They show that the phototemperature has very little effect upon SD induction; however, it would have been better if the SD induction period were limited to two instead of three days.

Nyctotemperature: In a single experiment a number of groups of plants were exposed to 18 LD followed by 2 SD (8-hour photoperiod in the 26° greenhouse plus a 16-hour nyctoperiod at various temperatures). At the end of SD induction the plants were returned to LD conditions for 5 days. The results of the experiment recorded in table II

Table II

The Effect of Temperature upon Short Day Induction

Temp.	% Fl **	% Term Fl	Nodes Fl/Pl *	No. of plants
		A. Phototemperature		
26	100	60	16 ± 4	5
23	100	100	20 ± 6	5
20	100	60	14 ± 4	5
17	100	80	18 ± 1	5
14	100	100	18 ± 1	5
10	100	50	16 ± 5	5
7	67	50	14 ± 4	6
		B. Nyctotemperature		
26	71	14	7 ± 3	7
23	86	43	14 ± 3	7
20	100	58	18 ± 2	7
17	100	29	11 ± 2	7
14	100	29	11 ± 2	7
10	0	0	0	7
7	0	0	0	7

* The errors quoted for the Nodes Fl/Pl represent the standard errors of the means.

** Abbreviations used: % Fl, percent flowering; % Term Fl, percent terminal flowering; Nodes Fl/Pl, number of flowering nodes per plant.

show the great effect of the nyctotemperature upon SD induction. The optimum nyctotemperature was approximately 20° and the minimum between 10 and 14°; within the range of temperatures employed there was no maximum.

Parker and Borthwick (5) working with Biloxi soybean, a SDP, have also shown that the effect of temperature was almost completely confined to the nyctoperiod. The differences between Cestrum and soybean are of a quantitative nature; there was a broad optimum nyctotemperature range for soybean between 21 and 32° (for Cestrum the optimum was 20°) and the minimum was below 7° (for Cestrum the minimum was between 10 and 14°). If the photosensitized reactions controlling floral initiation are the same in Cestrum and Biloxi soybean, these experiments show that there are differences in the associated thermochemical reactions which have a great effect upon the nyctotemperature requirements of SD induction. The results for Cestrum and soybean are in accord with one of the implications of the theory of Borthwick et al (1), discussed previously; namely, the nyctotemperature should have the greatest effect upon SD induction, for it is during the nyctoperiod that the pigment should be in the proper state (absorbing red radiation) for coupling with thermochemical reactions involved in the synthesis of the floral stimulus or its precursors.

Summary

The effect of temperature upon LD and SD induction in *Cestrum nocturnum*, a long-short day plant, has been investigated. It has been shown that the phototemperature has the greatest effect upon LD induction and that the nyctotemperature has the greatest effect upon SD induction. The implications of these results, particularly with regard to the "pigment" theory of Borthwick, Hendricks, and Parker, was discussed, and it was concluded that the facts for Cestrum are in accord with this theory.

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STUDIES ON TOBACCO ALKALOIDS. I. CHANGES IN NICOTINE AND NORNICOTINE CONTENT IN NICOTIANA[1,2]

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Since the publication of Nath's reports on the tobacco-tomato grafting (13, 14), numerous workers have employed similar techniques to study the biogenesis of tobacco alkaloids. However, these investigations did not result in any generally accepted interpretations until the publication of Dawson's work in 1941 and 1942 (2, 3, 4). At that time he stated "of all the organs of the tobacco plant, only the root possesses an appreciable capacity for synthesizing nicotine" (4). As a result of subsequent work (5) Dawson stated that "nornicotine is produced only in the plant leaf and at the expense of nicotine." In Mothes (11) recent review, evidence from a number of workers is cited which indicates that the synthesis, or breakdown, of tobacco alkaloids may not be as simple as these generalizations indicated.

Recent advances in experimental methods, particularly the use of paper chromatography (16), make possible the detection and separation of pyridine alkaloids when present in much smaller quantities than could be handled previously. It seemed desirable to re-examine the changes in the alkaloids during the development of the tobacco plant using these tech-

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niques. The results of this study as it concerns the major alkaloids are reported here, together with certain other experiments which may assist in the interpretation of the results obtained. The following paper, part II (17), reports on the formation of nicotine and nornicotine employing N^{15} in the solution culture of intact and grafted plants and of detached leaves.

Materials and Methods

Nicotiana glutinosa and *N. Tabacum* var. Robinson Medium Broadleaf Maryland tobacco were used in these experiments because previous studies had shown that they sometimes contained considerable quantities of nornicotine. Ten gm of seed of each species were ground in 2-gm portions to avoid difficulties due to the oiliness of the seeds. Each entire sample was then extracted successively with different selective solvents and the pyridine compounds in the extracts were separated by paper chromatography and estimated by ultraviolet absorption as described by Tso and Jeffrey (16) to determine the initial content of these substances.

Weighed quantities of seeds were germinated in Petri dishes on filter paper in the presence of distilled water and daylight. Seed and seedling lots were extracted at selected intervals with 50 % aqueous acetone (v/v) to follow the changes during germination and early growth. The results were calculated on the basis of weight of alkaloid present in the seeds or seedlings derived from a given initial weight of seed.

Plants for analysis at later stages were germinated in a sterilized sand flat, starting on September 24, 1953, and grown in the greenhouse in aerated solution culture, using McMurtrey's C_1 solution (10). Samples were taken at different time intervals until January 18, 1954. These plants were also extracted with 50 % acetone, taking into consideration the water content in the plant tissue; and the alkaloids were separated and estimated as before (16). In certain cases the values are reported as zero. This means that the substance was not detected on the paper by a technique capable of detecting 0.2 μgm, which with the usual size sample corresponded to about 1 ppm in the fresh tissue. Thus the upper limit possibly present per plant would depend on plant size.

Table I

Alkaloid Content in Seed of Nicotiana Tabacum Var. Robinson Medium Broadleaf and N. glutinosa

Extracts (16)	N. Tabacum		N. glutinosa	
	Substance	Quantity	Substance	Quantity
		mg/gm		*mg/gm*
Petroleum ether	None	0*	Nicotine	0.012
Chloroform	None	0	Unknown	0.023
Alcohol	None	0	Nicotinic acid	0.114
Water	None	0	Nicotinic acid	0.024
Total		0		0.175

* Substance not detected by technique used; less than 0.2 μgm.

Table II

Alkaloid Contents During Various Stages of Germination of Seed of Nicotiana Tabacum Var. Robinson Medium Broadleaf and N. glutinosa

Time interval	N. Tabacum		N. glutinosa	
	Substance	Quantity	Substance	Quantity
hrs		*mg/gm*		*mg/gm*
64	.....	...	Nicotine	0.45
72	None	0*	.....	...
88	.....	...	Nicotine	1.50
103	Nicotine	0.29	.....	...
168	Nicotine	10.76	.....	...

* Substance not detected by technique used; less than 0.2 μgm.

Results and Discussion

Alkaloids in the Seed and During Germination: Table I shows the results obtained from seeds of *N. glutinosa* and Robinson tobacco. No alkaloids or related compounds were detected in the seed of Robinson, but nicotinic acid, an unknown compound, and a very small amount of nicotine were found in the seed of *N. glutinosa*. The unknown had an R_f of 0.24 in the 1 : 1 *tert* amyl alcohol–pH 5.6 aqueous acetate buffer system used. Its absorption spectrum showed a minimum at 246 mμ and a maximum at 260 mμ. A yellowish-brown color was produced with PABA-CNBr (16). On the basis of these results it appeared to be a pyridine derivative. Since it appeared in the chloroform fraction it was evaluated as oxynicotine. It is generally accepted that commercial tobacco seed does not contain nicotine (6), though contrary views have been expressed (18). However, nicotine has been found in *N. rustica* seed by Schmid and Serrano (15) and by Mothes and Romeike (12). The different results of previous workers and in the present data may be due to (a) different species or varieties, (b) different stages of maturity of the seeds at harvest, (c) different storage conditions, and (d) different analytical methods, in the case of previous workers.

The increase in nicotine and related compounds during germination of the seeds in shown in table II. The results are expressed on the basis of one gram of air-dry seed. This seemed to be the only practical basis for estimating synthesis, as the weights of the seeds and seedlings were changing constantly.

The formation of alkaloids in *N. glutinosa* during germination was much more rapid than in Robinson tobacco. *N. glutinosa* seeds contained nicotinic acid which disappeared during germination and may have been used in nicotine synthesis or metabolized in other ways. Dawson (8) did not find any conversion of nicotinic acid–carboxyl C^{14} or of its ethyl ester into nicotine in tobacco but this does not exclude the possi-

bilities that such conversion might occur in a very young *N. glutinosa* seedling or that the carboxyl group from the nicotinic acid may have been replaced by a pyrrolidine group.

ALKALOIDS IN N. GLUTINOSA FROM SEEDLING TO MATURE PLANT: *N. glutinosa* seedlings were grown under two different conditions in the greenhouse. One test was grown using a sterilized white sand, while the other was grown using sterilized greenhouse soil. Table III shows the results obtained. Though dilute nutrient solution (one-half of the concentration used later in solution culture) was supplied to both lots of seedlings, growth conditions were evidently more favorable in soil than in sand. The fresh weight increased more rapidly in the plants growing in soil than in those growing in sand but the fresh weight at which a given alkaloid content was found was similar in the two groups. Therefore the alkaloid synthesis was more rapid in plants growing in soil, but this was an indirect result. Significantly nornicotine appeared either earlier or about the same time in the root as in the shoot. It seems very unlikely that nicotine was formed in the root, translocated to the leaf, converted to nornicotine, and then translocated back to the root at such an early stage of plant growth. In four experiments with Robinson tobacco grown in soil, nornicotine was either undeterminable in the shoots or present in lower concentration than in the root at first analysis. The percentages of nornicotine in the root on green-weight basis were 0.095, 0.074, 0.090, and 0.132, while in the shoot they were 0.032, 0, 0.016, and 0, respectively. In some instances the lower concentration in the shoot than in the root persisted for more than half of the growing period in this variety. Thus it seems highly probable that nornicotine can be formed in the root—at least in strains known to contain high proportions of nornicotine in the leaf at later stages. Nornicotine has not been detected in the roots of young plants of strains containing predominantly nicotine in the leaves throughout the season.

Since the majority of the alkaloid synthesis undoubtedly occurs in the finer roots, which cannot be recovered quantitatively in the case of plants grown in sand or soil, *N. glutinosa* seedlings were transplanted from the sand flat to solution culture on the thirty-third day after planting of the seed. This was the same day as the second sampling from the sand flat shown in table III, but the first harvest of material from solution culture was not made until a week after the last sampling from the sand flat. The solution used was half the concentration of McMurtrey's C_1 solution (10) used for the older plants. The results obtained with these plants are shown in table IV. It should be noted that the results, both as to fresh weight in grams and the milligrams of alkaloid, are expressed on a per plant basis rather than the per 100 plant basis used in table III. The nornicotine content of the shoot exceeds the nicotine content by the 61st day from seeding. By the 68th day the shoot had become large enough to be separated into stem and leaf samples and it is shown that the nornicotine was principally in the leaf portion.

After the plants had grown in the starter solution for two weeks some of them were transferred to full-strength culture solution, one plant per 2-gallon crock. The week following the last sampling of the starter-solution plants, or four weeks after the plants had been transferred to the full-strength solution, sampling of these plants was begun. They had been

TABLE III

DEVELOPMENT OF ALKALOIDS AND FRESH WEIGHT OF YOUNG SEEDLINGS OF NICOTIANA GLUTINOSA

DAYS AFTER SEEDING	FRESH WT (GM)	ALKALOID CONTENT (MG/100 PLANTS)*					
		WHOLE PLANT		ROOT		SHOOT	
		NIC.	NOR.	NIC.	NOR.	NIC.	NOR.
		Sterilized White Sand					
12	2	0.26	0**	....	....	....	....
33	20	3.36	0**	....	....	....	....
40	44	2.92	0.04	1.47	0.02	1.45	0.02
47	100	36.89	0.58	3.09	0.08	33.80	0.50
		Sterilized Soil †					
12	10	1.44	0**	....	....	....	....
19	20	3.38	0**	0.02	Trace††	3.36	0**
26	42	4.99	Trace††	0.12	Trace††	4.87	0**
33	83	11.22	0.44	1.82	0.03	9.40	0.41
40	116	31.39	2.43	4.18	0.08	27.21	2.35
47	170	62.68	20.17	5.56	0.09	57.12	15.08

* Analysis was made by using from 6 to 100 plants, depending on size.

** No color on paper.

† Roots from sterilized soil cannot be recovered completely.

†† Colored spots located in the nornicotine area by paper chromatography but not in sufficient quantity for accurate ultraviolet determination. Less than 0.01 mg.

TABLE IV

DEVELOPMENT OF ALKALOIDS AND FRESH WEIGHT PER PLANT OF NICOTIANA GLUTINOSA GROWN IN STARTER SOLUTION

TIME AFTER SEEDING	FRESH WT/ PLANT	ALKALOID CONTENT/PLANT PART							
		ROOT		SHOOT		STEM		LEAVES	
		NIC.	NOR.	NIC.	NOR.	NIC.	NOR.	NIC.	NOR.
days	*gm*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*
54	3.35	0.05	0.01	0.67	0.41	...	...	...	...
61	13.6	0.36	0.13	0.88	1.72	...	...	...	...
68	17.8	0.98	0.13	1.74	2.67	0.77	0.31	0.97	2.36

Plants transplanted into solution 33 days after seeding.

topped to 6 to 7 leaves for two weeks at that time and and were suckered frequently thereafter. The results are shown in table V.

In the case of plants at the stage of development represented in this table the major increase in alkaloid content is in the form of nornicotine in the leaves. There was also some increase in nicotine in the leaves at first, but this was reversed later, resulting in an amount too small to be determined by the techniques used. These techniques certainly would have detected 1 mg of nicotine in the approximately 150 gm of leaf material per plant, had this much been present, and probably would have detected considerably less.

There was also a noticeable increase in nicotine in the root and a much smaller increase in nornicotine there. A slow increase in nicotine occurred in the stem, but the nornicotine in the stem, always low, seems to have passed through a maximum at about the same time as the leaf nicotine reached a maximum.

It is very difficult to explain these data on the assumption that all of the nornicotine was formed in the leaf and translocated to the other parts. For example, in the week between these last two samplings, when nicotine was not determinable in the leaves, the amount of nornicotine in the leaves increased by more than 100 mg per plant. It would have to be assumed that twice as much nicotine was synthesized in the root during the last week as it contained at any one time, and that this occurred in a small fraction of the total root tissue. It would also have to be assumed that this nicotine passed through the stem without appreciable effect on the quantity there, though that quantity was only one-fourth as great, and the concentration was steadily falling due to growth of the stem. Most difficult of all, it must be assumed that this nicotine was immediately and quantitatively converted to nornicotine as it reached the leaf—even that portion which was in the conductive tissue, as the midribs of the leaves were included in the leaf samples analyzed. It is admitted that no incontrovertible proof has been demonstrated here of the synthesis of nornicotine without passing through nicotine. Nevertheless, in view of the data obtained on the young roots and the mature leaf an explanation based on the hypothesis of such a synthesis seems less taxing to the imagination than the older alternative.

On the other hand, if these data are examined while keeping in mind the limitations of the analytical methods available at the time the previous hypotheses were developed it is very easy to see how an investigator would conclude that the root and stem contained nicotine and the leaf contained nornicotine. Using picrate precipitation methods it is difficult to perceive, and impossible to estimate accurately, a minor alkaloid in the presence of a major one. Advances in understanding of physiological behavior can be made only as the tools of measurement become available.

No mention is made in the preceding five tables of compounds other than nicotine or nornicotine, though extensive evidence was obtained of the presence of other substances capable of forming colored spots on the paper with PABA-CNBr (16) and of absorbing ultraviolet light of the wave lengths absorbed most completely by pyridine compounds. Most of these compounds have not been identified and so cannot be assigned quantitative values, though the available evidence indicates that most of them are 3-pyridines. If their specific extinctions are of the same order of magnitude as those of nicotine and nornicotine the combined amount of these unknowns, in some cases,

TABLE V

DEVELOPMENT OF ALKALOIDS AND FRESH WEIGHT PER PLANT OF NICOTIANA GLUTINOSA GROWN IN CULTURE SOLUTION

TIME AFTER SEEDING	FRESH WT/ PLANT	ALKALOID CONTENT/PLANT PART					
		ROOT		STEM		LEAF	
		NIC.	NOR.	NIC.	NOR.	NIC.	NOR.
days	*gm*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*
75	100	8.2	1.6	9.8	1.1	13.3	49
82	149	14.0	2.1	14.9	1.7	21.4	145
89	142	12.3	3.0	16.8	1.5	36.2	156
96	176	22.0	3.1	14.1	2.3	38.3	224
103	242	52.1	2.6	14.8	1.9	19.1	262
110	245	45.4	3.1	23.6	1.1	0.0	327
117	299	57.0	4.4	24.2	0.9	0.0	430

Plants transplanted from starter solutions 46 days after seeding.

was equal to or even greater than the combined quantity of nicotine and nornicotine. The quantity of unknowns relative to the knowns was greatest in the root, intermediate in the stalk, and least in the leaf and tended to reach a maximum in the middle of the growth period and decrease in all three areas as maturity approached. More than ten spots were observed from certain samples. At the present time it is not known whether any of these compounds are precursors or decomposition products of the known alkaloids or were derived independently. Estimates of them could not be included in the preceding tables without confusing the results with respect to known compounds.

The results obtained on mature field-grown *N. glutinosa* plants, some of which had been allowed to flower and some of which had been topped and suckered, are shown in table VI expressed on the fresh-weight basis. In addition to nicotine and nornicotine, anabasine and myosmine could be identified and determined. Certain other substances which gave color with PABA-CNBr (16) and had an absorption maximum in the ultraviolet near that of the other 3-pyridine compounds could not be positively identified, and so an estimate of the sum of such substances is given using assumptions previously outlined (9). In this instance, also, nicotine was the principal alkaloid in the root and stem and nornicotine was the principal alkaloid in the mature leaves. Myosmine was found only in those samples which contained considerable amounts of nornicotine, which is in line with results on cured tobacco samples (9), some of which contained principally nornicotine with some myosmine and some principally nicotine and practically no myosmine.

ALKALOID COMPOSITION OF ROBINSON TOBACCO: A number of different experiments have been performed which provide contributory evidence to that presented above.

The Transformation of Nicotine to Nornicotine in Different Parts of Plants: There is little doubt that nicotine demethylation is one of the principal sources of nornicotine formation. This experiment was designed to determine in which part of the plant this process takes place. This can be done more unequivocally when the parts of the plant are detached from each other.

TABLE VI

ALKALOIDS IN MATURE GREEN PLANT OF NICOTIANA GLUTINOSA

ALKALOIDS	NOT TOPPED OR SUCKERED			TOPPED AND SUCKERED		
	ROOT	STEM	LEAF	ROOT	STEM	LEAF
	% alkaloid on green wt basis					
Nicotine	0.165	0.038	0.031	0.191	0.133	0.017
Nornicotine	0.006	0.008	0.095	0.013	0.017	0.853
Anabasine	0.005	0.003	0.006	0.006	0.003	0.036
Myosmine	0*	0*	0.002	0*	0*	0.036
Others	0.016	0.007	0.017	0.026	0.007	0.121
Total	0.192	0.056	0.151	0.236	0.160	1.063

* Less than 0.001 %.

TABLE VII

THE TRANSFORMATION OF NICOTINE TO NORNICOTINE DURING AIR-DRYING IN ROBINSON MEDIUM BROADLEAF TOBACCO

PLANT PART	ALKALOID CONTENT/PLANT PART					
	ORIGINAL			AFTER 1-WK AIR-DRYING		
	TOTAL*	NICOTINE	NORNICOTINE	TOTAL*	NICOTINE	NORNICOTINE
	mg	*mg*	*mg*	*mg*	*mg*	*mg*
Root	56	45	9	45	35	8
Stem	43	34	6	32	29	3
Leaf	230	179	47	183	26	142

* Including alkaloids other than nicotine and nornicotine.

Previous studies in this laboratory have shown that, unlike *N. glutinosa,* the transformation of nicotine to nornicotine in Robinson tobacco occurs principally during senescence. Starting from the bottom of two mature plants, even-numbered leaves from plant A and odd-numbered leaves from plant B were removed and a composite of these leaves was analyzed immediately to determine the original alkaloid content. The other leaves, odd-numbered from plant A and even-numbered from plant B, were hung in the greenhouse after tying each leaf to a stick with string. These leaves were analyzed one week later. Stems and roots were split in half longitudinally and one-half of similar parts from each plant was used for immediate analysis, the other halves were hung in the greenhouse. The results in table VII show an apparent decrease of total alkaloid, which is usual during air-curing. The observed decrease in nornicotine was slightly less proportionally in the root and considerably greater in the stem than the decrease in nicotine. However, the only safe conclusion from this experiment is that no positive evidence was found of the conversion of nicotine to nornicotine in the root or stem. On the other hand, evidence of the increase of nornicotine at the expense of nicotine in the leaf is clear. This agrees with Dawson's statement (7) that only intact green leaves were able to conduct this transformation. Thus it appears that the nornicotine found in the root was probably produced by synthesis rather than by degradation. The sum of the nicotine and nornicotine in the dried leaf was 15 mg less than the total alkaloid value, indicating a much larger quantity of degradation products in this sample than in any other.

The Content of Nicotine and Nornicotine at Different Leaf Positions: The Robinson tobacco plant used in this experiment was grown in the greenhouse in solution culture. Six days after topping the leaves were carefully removed in order, starting from the

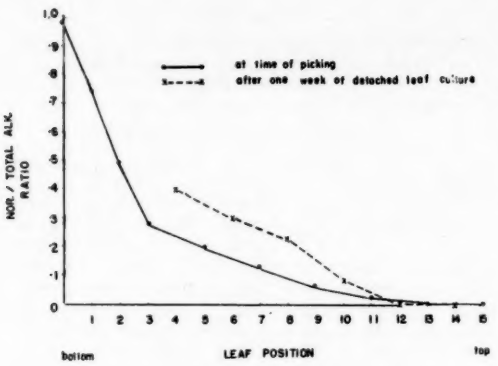

FIG. 1. Nornicotine/total alkaloid ratio at different leaf positions.

bottom of the plant. Leaves number 4, 6, 8, 10, 12, and 14 were subjected to one week of detached leaf culture, using the methods described by Dawson (1). The remaining leaves were immediately analyzed individually for total alkaloid, nicotine, and nornicotine. The detached leaf culture leaves were analyzed similarly one week later. The concentration of total alkaloid on an initial fresh-weight basis was essentially constant from the bottom to the top of the plant and was unchanged by a week of leaf culture. Thus the results can be shown most clearly when expresesd as the proportion of the total alkaloid which is nornicotine. These results, shown in figure 1, indicate that the mature basal leaves, when analyzed at the same time, contain more nornicotine and less nicotine than the immature upper leaves. The nornicotine content in the lowest leaf reached 98 percent of the total alkaloid content, while the top one contained no detectable nornicotine. The detached leaves, which had an additional period of one week for nicotine transformation, show an increase of nornicotine content up through the tenth leaf but still have a higher proportion of nornicotine in the older leaves than in the younger. Evidently the system, presumably enzymatic, by which nicotine is converted to nornicotine had not become active in the most immature leaves.

The Effect of Topping and Suckering on the Conversion of Nicotine to Nornicotine: The nornicotine content in Robinson tobacco differs greatly from season to season. Since the great majority of the nornicotine is derived from nicotine in strains such as this in which large quantities of nornicotine do not appear until late in the season, it is evident that the nornicotine content is influenced by the amount of nicotine synthesized, as well as by the proportion which is converted to nornicotine. Experiments have been conducted in which the influence of moisture, temperature, and topping practices on the conversion of nicotine to nornicotine have been observed. Of these variables, only topping appears to have a consistent influence. The results of some of these experiments are shown in table VIII. Plants were grown in pots during two seasons of the year and in two greenhouse sections each time. Attempts were made to keep one of these sections at 75° F (24° C) night and day and the other at 50 to 65° F (10 to 18° C) at night and 65 to 75° F (18 to 24° C) in the daytime. These conditions could be maintained fairly well during the experiment extending from October to February, but during the previous experiment ex-

TABLE VIII

THE EFFECT OF TOPPING AND SUCKERING AND OF TEMPERATURE ON THE ALKALOID COMPOSITION AND PLANT SIZE OF ROBINSON MEDIUM BROADLEAF TOBACCO

TRANSPLANTING DATE	GREENHOUSE SECTION	PLANT PART	NOT TOPPED OR SUCKERED					TOPPED AND SUCKERED				
			HARVEST FRESH WT	NICOTINE	NORNICOTINE	TOTAL ALKALOID	NORNIC. ×100/ TOTAL ALK.	HARVEST FRESH WT	NICOTINE	NORNICOTINE	TOTAL ALKALOID	NORNIC. ×100/ TOTAL ALK.
			gm	*mg*	*mg*	*mg*		*gm*	*mg*	*mg*	*mg*	
Mar. 22, 1954	Warm*	Root	83	53	8	64	13	90	156	22	182	12
		Shoot	859	100	236	499	47	675	1188	259	1586	16
	Cool**	Root	95	59	16	75	22	148	128	28	168	17
		Shoot	657	161	215	426	50	482	742	553	1438	38
Oct. 20, 1954	Warm†	Root	32	27	7	35	19	73	94	15	114	13
		Shoot	399	190	65	270	24	450	838	140	989	14
	Cool††	Root	52	37	9	47	18	78	154	24	190	13
		Shoot	380	76	30	110	27	313	800	251	1130	22

* Intended temperature 75° F (24° C) day and night, but often warmer in daytime during the latter part of the growing period. Harvested May 18, 1954.

** Intended temperature 65 to 75° F (18 to 24° C) daytime, 50 to 65° F (10 to 18° C) at night but often warmer in daytime during the latter part of the growing period. Harvested June 3, 1954.

† Intended temperature same as warm section of spring experiment but more nearly maintained. Harvested Jan. 19, 1955.

†† Intended temperature same as in cool section of spring experiment but more nearly maintained. Harvested Feb. 14, 1955.

tending from March to June the temperature of both sections rose above the intended levels at times during the latter part of the experiment and the difference between warm and cool sections was not maintained.

The growth of the plants was more rapid in the warm section, necessitating the topping of these plants on April 19 and November 29, whereas the plants in the cool section were not ready to top until May 6 and December 12. Similar differences in harvest dates occurred, as shown in the table, but the fresh weight of plant obtained and the amount of alkaloid found in both root and shoot was not significantly different due to temperature in the two sections so long as extra growth time was given to the plants in the cool section. On the other hand, the average amount of total alkaloid in the root of the topped plants is over three times as high as in the corresponding untopped plants and in the shoot over five times as high. The amount of nornicotine found in the topped plants is also higher than in the corresponding part of the untopped plants, but the difference in nornicotine content is not as great as the difference in total alkaloid content. Thus, the ratio of nornicotine to total alkaloid content is significantly lower in the topped plants than in the untopped plants.

Completely satisfactory evidence does not exist at present as to the chemical mechanism of the conversion of nicotine to nornicotine, i.e., whether a demethylation or a transmethylation is involved. Similarly, unequivocal evidence is not available concerning the possibility of one or more enzyme systems being involved. However, if the concersion is enzymatic and the activity of the enzyme system is limiting the rate of nornicotine formation, one would expect to find a relation of substrate quantity to degree of conversion such as here observed.

It should be noted, however, that different species or strains have different ability for nicotine-nornicotine conversion. *N. glutinosa* shows a much higher power of such conversion than does Robinson tobacco. As shown in table VI, the topped and suckered *N. glutinosa* plant has seven times more total alkaloid than that not topped or suckered, while the nornicotine to total alkaloid ratio is even higher in the leaf of the topped than the untopped plants. These results indicate that the activity of the enzyme system which is responsible for nicotine–nornicotine conversion in *N. glutinosa* is much greater than in Robinson tobacco, and therefore an increase in substrate would not affect appreciably the amount converted. Also, since topped plants had a higher proportion of mature leaves than did the untopped plants, a higher nornicotine to total alkaloid ratio is to be expected in the former than the latter if the enzyme is not a limiting factor.

Summary

The content of nicotine and of nornicotine in two selections from the genus Nicotiana, known to contain more nornicotine than most commercial varieties of tobacco, was observed at different stages from seed to mature plant.

No pyridine alkaloids were found in the seeds of Robinson tobacco, while nicotinic acid and nicotine were detected in the seeds of *N. glutinosa*. The initial rate of nicotine synthesis was much higher in *N. glutinosa* than in Robinson tobacco. In young seedlings of both strains nornicotine was detected in the root as early or earlier than in the shoot. From *N. glutinosa* seedlings to mature plants the nornicotine content per plant increased continuously, but nicotine increased and then decreased. No nicotine was detected in the leaves of over-mature, greenhouse-grown *N. glutinosa* during the last two weeks when the nornicotine increased 62 and 103 mg per plant weekly. There was little change of nicotine and nornicotine content in the root or stem at this time. All these results indicate that some nornicotine found in the root or in the shoot may not be formed from nicotine.

In Robinson tobacco the conversion of nicotine to nornicotine is not accelerated to the same degree as a result of topping as is nicotine synthesis, thus the ratio of nornicotine to total alkaloid is lower in topped plants. In *N. glutinosa*, where the nicotine to nornicotine conversion system is strongly active even in immature plants, this system is not limiting even when the alkaloid content is increased greatly due to topping and suckering.

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UTILIZATION OF GLYCEROL IN THE TISSUES OF THE CASTOR BEAN SEEDLING [1, 2]

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Although it has been recognized for some time that glycerol, as one of the products of lipase action, is produced during the germination of fatty seeds, no definitive work seems to have been done on its subsequent metabolism. The present experiments with glycerol-1-C^{14} were begun in the hope that the use of labeled material might allow a successful approach to this problem.

While this work was in progress Stumpf reported an investigation of glycerol oxidation by cell-free preparations from peanut seedlings (11). He succeeded in providing evidence for the existence of enzyme systems which cooperated in converting glycerol to CO_2 by a pathway including α-glycerol phosphate and pyruvate and subsequent oxidation in the Krebs cycle. Although the amounts of glycerol oxidized by the cell free system were very small, the results gave firm support for the conclusion that an oxidation pathway which might have appeared the most likely from a knowledge of comparative biochemistry did in fact exist in the tissue concerned.

All of the experiments in the present report were carried out on intact tissues. It will be shown, however, that there is evidence for the participation of a sequence of reactions, analogous to that described by Stumpf, in the very active oxidation of glycerol by castor bean cotyledons. In addition, it becomes clear that oxidation to CO_2 represents only one of the metabolic fates of glycerol in the tissues; a considerably larger fraction of the supplied glycerol is rapidly incorporated into sucrose. Preliminary reports of this work have appeared elsewhere (2, 4).

MATERIALS AND METHODS

Castor bean seeds (*Ricinus communis* L.) var. U.S. 70 were germinated as described elsewhere (5). Under these conditions the seedling produces a primary root of about 8 cm in 4 days, when the hypocotyl is just beginning to elongate. After 7 to 8 days the hypocotyl has reached a height of about 10 cm and the endosperm tissue has by then been almost completely absorbed by the enlarging cotyledons. It has been shown (4, 10) that active hydrolysis of the fats in the seed begins after 3 to 4 days of germination and free sugars, particularly sucrose appear in quantity in the seedling. Most of the experimental work in the present paper was done with cotyledons taken from 5- to 6-day seedlings; i.e., at a stage when an active breakdown and utilization of fats in the endosperm was occurring. Some experiments with other tissues of the seedling are also included, and for comparison the ability of some other tissues to oxidize glycerol was also examined.

Glycerol-1-C^{14} with a rated specific activity of 1 millicurie per millimole was supplied by California Foundation for Biochemical Research. For use it was diluted with an unlabeled glycerol solution so that 0.1 ml contained 10 micromoles and from 1,000 to 6,000 cpm (counts per minute). The actual activities of the glycerol solutions used in the individual experiments were determined by oxidation in the apparatus of Stutz and Burris (12).

The methods used are similar to those which have been described previously in another connection (6). The intact cotyledons (or thin slices of the other tissues) were placed in 100-ml Warburg flasks with 3 to 4 ml solution containing 0.5 ml 0.1 M potassium phosphate at pH 5.0. The CO_2 was collected in KOH, converted to $BaCO_3$ and assayed for radioactivity in a windowless gas flow counter. The figures for radioactivity are recorded in cpm, corrected for background and self-absorption.

At the end of the experimental period the tissues were extracted in hot 80 % ethanol. After evaporation of the water and alcohol on a steam bath the residue was washed with ether and then dissolved in water. The solution was treated with Amberlite MB-1 exchange resin (Rohm and Haas) to remove ionic materials, and concentrated to a small volume for application as a band on Whatman No. 3 paper. It was chromatographed for 24 hours with *n*-butanol-ethanol-water (52.5:32:15.5 v/v) as the solvent. The solvent was allowed to drip off the paper. After drying, the paper was cut crosswise into ½″ strips.

[1] Received May 8, 1956.

[2] This paper is based on research supported by a grant (NSF-G 265) from the National Science Foundation.

The sugars were eluted by suspending the individual strips on hooked "cold fingers" in test tubes containing about 3 ml of water and refluxing for 30 to 40 minutes. The eluates were made to 5 ml and 2 ml samples were taken from each for sucrose analysis by the orcinol method (7). A further 2-ml sample from each tube was dried down on a steel planchet and counted directly. Hydrolysis of the sucrose was accomplished with a commercial invertase preparation (Nutritional Biochemicals Corporation) and the separation of glucose from the products carried out as in table IV.

Degradation of the glucose was carried out by the method of Gunsalus and Gibbs (8).

Results

Respiration of Glycerol by Plant Tissues: The results in table I show that glycerol is converted to CO_2 by each of the materials tested, but at widely different rates; several tissues without fatty food reserves nevertheless attack glycerol actively. For a given weight of tissue, the castor bean cotyledon was the most efficient in its ability to oxidize glycerol but this is also the tissue with the highest metabolic rate as shown by the rate of endogenous CO_2 release. The addition of small amounts of glycerol did not lead to increases in the already high endogenous rates of O_2 uptake and CO_2 output. It will be noticed that the castor bean endosperm tissue, which may be regarded as the source of glycerol, was much less efficient in oxidizing it than the neighboring tissues (cotyledon and hypocotyl) of the seedling proper.

Course of $C^{14}O_2$ Release and $C^{14}O_2$ Yield During Glycerol Oxidation by Castor Bean Cotyledons: Figure 1 shows that, within the range 10 to 30 micromoles, the total yield of $C^{14}O_2$ depends on the amount of substrate supplied. At each substrate level, the initially high rate of $C^{14}O_2$ evolution declined rapidly, even though the rate of endogenous CO_2 output was maintained. The progress curves for percentage yield follow a closely similar course throughout, and show that at each level of glycerol roughly 25 % of the substrate provided had appeared

Table I

The Relative Abilities of Tissues to Oxidize Glycerol

Tissue	Mg $BaCO_3$ produced	Percent of supplied radioactivity in respired CO_2
	Castor bean, 6-day	
Endosperm	6.0	0.2
Petiole	8.2	1.7
Hypocotyl	14.4	6.0
Cotyledon	29.4	14.4
	Tomato	
Internode	6.9	0.7
	Corn	
Stem	5.2	1.5
Root	6.9	1.7
	Pea	
Internode	6.0	4.1
	Red kidney bean	
Petiole	8.1	7.7

One gm samples of the various tissues were allowed to respire for 4 hrs in a medium containing 20 micromoles glycerol-1-C^{14}. The respired CO_2 was collected in KOH and converted to $BaCO_3$.

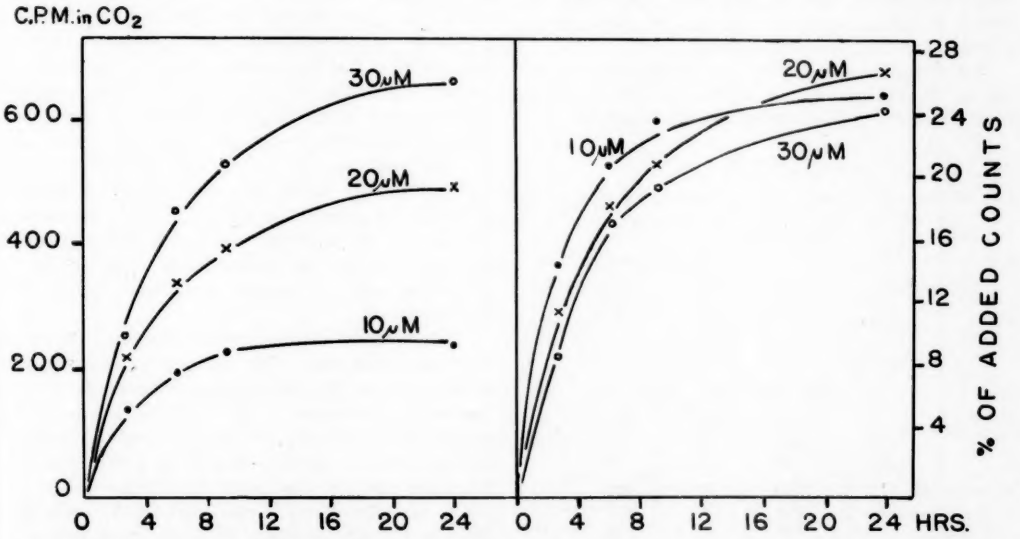

Fig. 1. Time course of glycerol oxidation by castor bean cotyledons. One gm samples were incubated with the amounts of glycerol indicated, in 4.0 ml solution containing 1.0 ml 0.1 M KH_2PO_4, pH 5.0.

as CO_2 at the end of 23 hours, when the rates of $C^{14}O_2$ release had slowed down to a low value.

This evidence might be taken to indicate that a major fraction of the supplied glycerol suffers an alternative fate and subsequent experiments have shown this to be so. Most of these experiments have been of 4- to 6-hr duration and the amount of glycerol oxidized during this period was usually in the range of 15 to 20 % of that added ("control" values in table V).

Pathway of Glycerol Oxidation: Some clues as to the mechanism of the glycerol-CO_2 conversion in vivo are provided by the following considerations.

(a) Anaerobic conditions almost completely prevent the breakdown of glycerol to CO_2. Whereas in air, in a particular experiment, 20.4 % of the added glycerol-C^{14} had appeared as CO_2 after 4.75 hours, the percentage yields from duplicate samples in nitrogen were 1.68 and 1.43; a reduction of some 92 % due to removal of O_2 from the system. Fermentation of endogenous substrates was, nevertheless, quite high (CO_2 output 64 % of that in air). It thus appears likely that the hindrance to glycerol breakdown imposed by anaerobic conditions is not due to any attendent deficiency in phosphorylating capacity of the tissue. One might, rather, suppose that some oxidative step in glycerol utilization is obligatorily linked to atmospheric O_2 and thus cannot be maintained by the acetaldehyde to alcohol reduction which is recognized as the major internal H-accepting system under these conditions. Although these considerations do not rule out the possible participation of a DPN-linked α-glycerophosphate dehydrogenase they would be neatly accommodated, if, as in Stumpf's system, the dehydrogenation of α-glycerophosphate by the cytochrome-linked enzyme (which would require O_2 for its continued operation) afforded the main pathway for the introduction of glycerol into the glycolytic sequence.

(b) Fluoride slows down the endogenous O_2-uptake of the cotyledons and inhibits the $C^{14}O_2$ release from glycerol-C^{14} to a similar degree (table II).

Table II

The Effects of Fluoride and Malonate on O_2 Uptake and $C^{14}O_2$ Output in the Presence of Glycerol-1-C^{14}

Inhibitor		O_2 uptake	% of control	$C^{14}O_2$ output	% of control
Type	Conc.				
	$M \times 10^{-2}$	μl		*cpm*	
0	0	2150; 2300	100	1065; 1087	100
NaF	5	1013	46	477	44
"	13	175	8	54	5
"	25	16	1	8	1
Malonic acid	13	895	40	1550	143
" "	25	458	21	697	65
" "	50	326	15	525	49

Samples (500 mg) of cotyledons were incubated for 6.75 hrs with 20 micromoles glycerol-1-C^{14} (10,800 cpm).

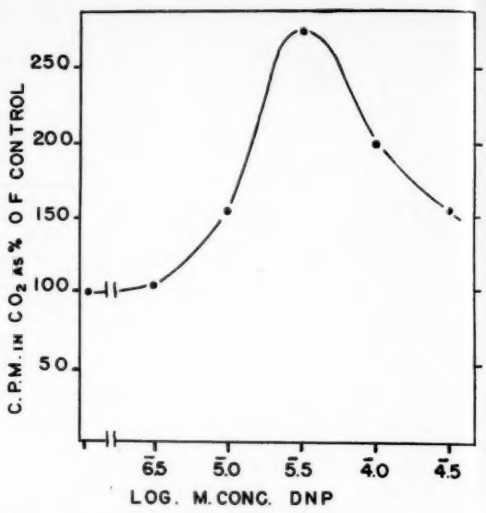

Fig. 2. The effect of 2,4-dinitrophenol on $C^{14}O_2$ release from glycerol. Samples of 800 mg cotyledon tissue were incubated for 5 hrs in the presence of 20 micromoles (4470 cpm) of glycerol-C^{14}.

(c) Malonate also reduces $C^{14}O_2$ output, but only in concentrations greater than 0.02 M. However, O_2-uptake was much more strikingly depressed, and at the lowest malonate level, which actually stimulated $C^{14}O_2$ output, O_2 uptake was reduced by more than half. From other experiments with malonate it has been concluded that when the operation of the Krebs cycle is slowed down by malonate, pyruvate is diverted to CO_2 and alcohol (1) and the CO_2 output (which under these conditions results from the residual Krebs cycle activity and the decarboxylation of pyruvate to acetaldehyde) may be much less inhibited than the O_2 uptake. If this were so in the present case then a fraction of the $C^{14}O_2$ produced from glycerol in the presence of malonate might have arisen from direct pyruvate decarboxylation. The fact that radioactive alcohol, with 97 % of its activity in the CH_3 group, has now been isolated from cotyledons respiring glycerol-1-C^{14} in the presence of malonate is obviously in accord with this supposition, and may, in fact be considered as evidence for the participation of pyruvate as an intermediate in glycerol oxidation.

The Effect of 2,4-Dinitrophenol (DNP) on Glycerol Oxidation: The addition of DNP brought about effects of the kind shown in figure 2. At concentrations between 6×10^{-6} and 1×10^{-3} M, $C^{14}O_2$ output from glycerol-C^{14} was strikingly stimulated, and at about 4×10^{-5} M the yield of $C^{14}O_2$ was some 2.5 times that of the control. In the absence of DNP, as pointed out earlier, the $C^{14}O_2$ yield from glycerol amounts to about 20 % of that supplied; at the most effective DNP levels, yields of up to 73 % of the supplied glycerol have been obtained.

IDENTIFICATION OF SUCROSE AS A MAJOR PRODUCT OF GLYCEROL UTILIZATION: When extracts from untreated cotyledons were chromatographed and the papers sprayed for reducing sugars before and after hydrolysis with invertase, it was at once clear that sucrose comprised by far the greatest proportion of free sugars. Only faint traces of glucose and fructose were observed. Extracts from tissues which had been incubated for several hours with glycerol-C^{14} contained similarly large amounts of sucrose (up to about 100 micromoles/gm fresh weight in 7-day cotyledons) and direct counting on the paper showed that the sucrose area was radioactive. An autoradiograph showed that other areas of the chromatogram were virtually without activity.

At the end of 6- to 7-hr experiments in which 1 gm of cotyledons had been incubated with 20 micromoles glycerol-C^{14}, it was found that the cotyledon residues, after 80% alcohol extraction, contained only small amounts of activity as judged by a thin window counter. The ether washings contained less than 10% of the added C^{14}. Thus the material placed on the chromatogram contained the bulk of the C^{14} which had not been respired as CO_2, and since this appeared to be concentrated in the sucrose area it was clear that a substantial fraction of the glycerol had been incorporated into this product. Quantitative estimation was therefore undertaken by the methods outlined above.

The results obtained from a typical chromatogram are shown in figure 3. The coincidence of the sucrose and radioactivity peaks is striking. The smaller peak in radioactivity in samples 11 and 12 corresponds to unreacted glycerol. Any radioactive glucose or fructose would have given rise to peaks in the region of samples 6 to 10. Estimates of total sucrose and radioactivity were obtained by summing the contributions of samples comprising the sucrose peak of the curve; in the example shown these would be samples 3 and 4.

The "control" values in table V are examples of such estimates and these show that as much as 60% of the glycerol carbon supplied may be incorporated into this sucrose component, and at the same time somewhat less than 20% of the C^{14} was appearing as CO_2.

After chromatographing again in the same solvent and eluting, a sample of the radioactive sucrose was partially hydrolyzed with invertase and the products separated chromatographically in phenol-water. The following amounts of radioactivity were observed in the products: unhydrolyzed sucrose, 420 cpm; glucose, 968 cpm and fructose, 843 cpm. It is clear therefore that the glucose and fructose moieties of the sucrose are fairly equally labeled.

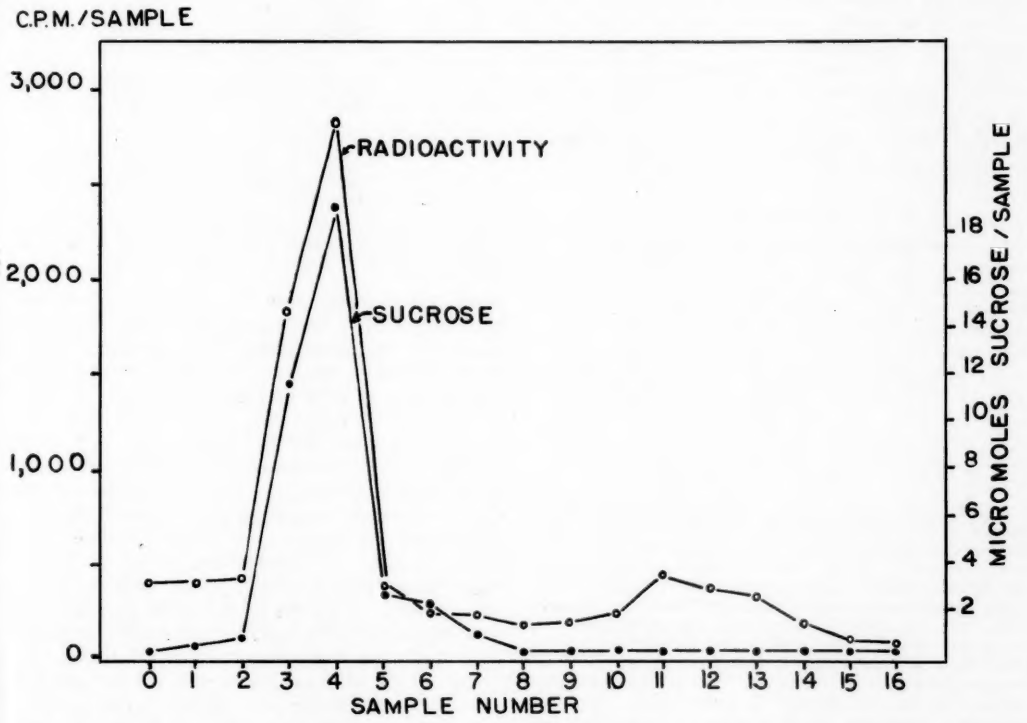

FIG. 3. Sucrose content and radioactivity of eluates from successive ½" strips cut from a typical chromatogram (see text).

Table III

The Effect of Added Glycerol on the Sucrose Content of 7-Day Cotyledons

Conditions	Glycerol added	Sucrose/gm fresh wt of tissue
	micromoles	*micromoles*
Beginning of experiment	0	95.2
After 6-hr incubation	0	87.0
" " "	50	91.0
" " "	400	96.4

As shown in table III there is a considerable utilization of native sucrose in the cotyledon during the experimental period. The provision of the small amounts (20 micromoles) of glycerol used in the above experiments does not lead to any net synthesis of sucrose, although a sparing effect on the utilization of sucrose is evident. In the presence of larger amounts of glycerol, however, the sucrose deficit may be completely offset and even a small amount of net synthesis obtained (table III). Although the endosperm, in contrast to the cotyledon, was found to oxidize glycerol only very slowly, a single experiment showed that there is a rapid assimilation of glycerol into sucrose in this tissue also.

The large percentage incorporation of glycerol carbon into sucrose would indicate that the intact C-3 skeleton, rather than any smaller breakdown product, was being assimilated. That this is, in fact, so was shown by degrading stepwise a glucose sample obtained from hydrolysis of the radioactive sucrose by the procedure outlined in table IV. The last row of figures shows that 97 % of the C^{14} was confined to carbons 1, 3, 4 and 6 of the glucose, and distributed fairly evenly between these positions. Such a distribution of activity would be predicted only if the terminally labeled glycerol skeleton was incorporated intact into the glucose.

Table IV

Isolation and Degradation of Glucose

Carbon atom of glucose	Specific activity $M\mu C/mg\ C$	Activity as % of total
1	1.66	26.8
2	0.09	1.5
3	1.58	25.3
4	1.51	24.4
5	0.11	1.8
6	1.23	20.0

Castor bean cotyledons were incubated with glycerol-1-C^{14} and extracted with hot 80 % alcohol. The extract was evaporated to dryness, washed with ether and dissolved in water. After treatment with Amberlite MB 1, it was chromatographed on Whatman No. 3 paper in *n*-butanol-ethanol-H_2O (solvent I). The sucrose area was eluted and chromatographed again in the same solvent. After elution the sucrose was hydrolyzed with invertase and the products separated chromatographically using solvent I. The glucose area was eluted and chromatographed in phenol-H_2O. The glucose (80 micromoles, 12,500 cpm) was eluted and 400 micromoles carrier glucose added before degradation by the Leuconostoc procedure (8).

Effects of Interfering Substances on the Incorporation of Glycerol into Sucrose, *Dinitrophenol:* DNP in the same concentrations which stimulate glycerol oxidation produces equally striking and opposite effects on the conversion of glycerol to sucrose. As the data in table V show, the reduction in assimilation is offset in each case by a corresponding increase in CO_2 output from the glycerol and the total C^{14} accounted for in the two fractions (60 to 80 % of that added) was essentially the same whether DNP was present or not.

Malonate and Fluoride: Malonate and fluoride in concentrations which strongly inhibited glycerol oxidation, also slowed down its incorporation into sucrose, but to a somewhat lesser degree. Thus when the fluoride (0.07 M) and malonate (0.07 M) inhibitions of $C^{14}O_2$ output were 97 % and 84 %, respectively, the incorporation of glycerol-C^{14} into sucrose was reduced by 82 % and 69 %.

Table V

The Effect of Dinitrophenol on the Fate of Glycerol Supplied to Castor Bean Cotyledons

Conc DNP $M \times 10^{-5}$	Cpm in CO_2	% of added activity	Cpm in sucrose	% of added activity
	Expt I—4 hrs			
0	1800	15.3	5086	43.0
1.5	4040	34.3	3230	27.7
3.0	6450	54.7	1766	15.0
5.0	5250	44.5	1658	14.1
	Expt II—6 hrs			
0	2152	18.1	7177	60.4
2.9	5920	49.7	3644	30.6
	Expt III—5 hrs			
0	890	19.9	2030	45.6
2.9	2360	52.7	734	16.4

To each sample 20 micromoles of glycerol-1-C^{14} were added. Sucrose isolated from the tissue and the respired CO_2 were assayed for radioactivity.

Discussion

The results show that, at least in the excised castor bean cotyledon, the bulk of the glycerol supplied to it suffers one of two fates. One of these, incorporation into sucrose accounted for about three times as much of the glycerol carbon as did the other, conversion to CO_2. These changes are accomplished in a few hours and after this there is a slow release of $C^{14}O_2$, which presumably originates from the radioactive sucrose pool in the tissues.

The effects of anaerobiosis and of the inhibitors malonate and fluoride were shown to be those expected if the glycerol were being oxidized by a sequence involving introduction of a phosphorylated

glycerol derivative into the glycolytic sequence and oxidation of the resulting pyruvate through the Krebs cycle. Such a sequence which had seemed likely from considerations of comparative biochemistry has in fact recently been demonstrated in a cell-free system from peanut seedlings by Stumpf (11). The present demonstration that the pattern of labeling in the glucose (derived from the sucrose by hydrolysis) is such as to indicate that two glycerol skeletons have condensed together is strong presumptive evidence that triose phosphates are also intermediates in the synthesis of sucrose from glycerol.

It is therefore supposed that the metabolism of glycerol in the cotyledon involves conversion to triose phosphate and that this may be the point at which the two major pathways of its utilization diverge. Since each molecule of sucrose synthesized represents four glycerol residues, and the incorporation of glycerol carbon into sucrose was about 3 to 4 times that of its appearance in the CO_2 it is clear that under the conditions of experimental glycerol supply, the rates of the reactions which competed for the triose were fairly similar. In the presence of DNP, it will be recalled, there were striking changes in the relative contributions of the synthetic and oxidative pathways of glycerol utilization (table V). The increased oxidation of the glycerol is thought to be due to the indirect induction of a higher rate of glycolysis by the DNP (3). Under these conditions glycolysis competes on superior terms for the triose intermediate and thus a much smaller fraction of the glycerol is converted into sucrose. This interpretation would give a further point of emphasis to the importance of the control exerted over the glycolysis rate by the "aerobic" phosphorylative events which are subject to uncoupling by DNP.

Finally, it should be mentioned that the ability of the castor bean tissues to convert smaller molecules into sucrose is not confined to glycerol, since a large fraction of supplied acetate carbon may also be so incorporated (unpublished results). These reactions whereby each of the presumptive breakdown products of fat may be converted to sugars are apparently of some importance in the economy of the castor bean seedling, since it has been shown that conversion of fatty reserves into carbohydrate takes place in this material (10) and the respiratory quotients of those parts of the embryo other than the cotyledons indicate that carbohydrates alone are being respired (9).

Summary

Non-green cotyledons excised from young germinating seedlings of castor bean have been shown to metabolize glycerol-C^{14} actively under aerobic conditions. About one quarter of the supplied glycerol was oxidized to $C^{14}O_2$ by a sequence which apparently involved glycolytic intermediates, pyruvic acid and the Krebs cycle. A large fraction of the remainder of the glycerol was found in sucrose isolated from the tissue, and degradation showed that the glycerol carbon skeleton had been incorporated intact, as would be required if the glycerol were first converted to triosephosphate and then subject to the action of aldolase.

The effects of 2,4-dinitrophenol on this system are described, and the interpretation put on these results is that the extent to which synthesis of sucrose from glycerol occurs is governed by the rate of glycolytic utilization of triosephosphate, which is itself regulated by the aerobic phosphorylations in the tissue.

I am indebted to Dr. Martin Gibbs for instructing me in the use of the Leuconostoc degradative procedure.

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THE FATE OF 2,4-DICHLOROPHENOXYACETIC ACID IN BEAN SEEDLINGS. II. TRANSLOCATION[1]

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Reports on the translocation of 2,4-dichlorophenoxyacetic acid (2,4-D) are confused and contradictory. Because the transport of indoleacetic acid (IAA) is known to be polar and basipetal, it might be expected that 2,4-D applied as a herbicide would be transported in the same way, and some workers have assumed this. Yet it seems inherently improbable that the mechanism of normal auxin transport, which is dependent on metabolism, would operate with a substance whose concentration is high enough to poison the cells it enters. Other workers, observing that the application of isotope-labeled 2,4-D gives rise to radioactivity in all parts of the plant, have concluded that transport takes place readily in all directions. It was shown in part I, however, (7) that deductions based merely on isotope distribution after application of 2,4-D are invalid. Thirdly, a number of experiments have been considered to indicate mass flow of 2,4-D in the phloem, while many field observations point only to movement with the transpiration stream in the xylem.

For these reasons, a study of the translocation of 2,4-D at moderate concentrations, under controlled laboratory conditions, and by a method specific for the intact 2,4-D molecule seemed desirable. The method used is described in the preceding paper (7). The plants were seedlings of *Phaseolus vulgaris*, var. Dwarf Red Kidney bean, 14 days old, with their roots in water and all leaves but one removed. Experiments were carried out under artificial light (about 1900 ft-c) at 19° C unless otherwise described in the text. With this method, the transport and loss of 2,4-D were followed simultaneously. The material was applied to the one remaining primary leaf and later extracted from various parts of the plant. The amounts recovered were assayed with the split pea-stem test to yield quantitative data on the amounts of unchanged 2,4-D moving to each part of the plant under various conditions. All figures are the average of at least two trials with the result that in some cases data in the tables may be slightly below the minimum determinable in a single test (7). Particular attention was given to the quantities moving to the base of the shoot and to the roots, since this information is not given by methods which depend on the curvature of the epicotyl, or on the inhibition of the terminal bud of seedlings to which 2,4-D has been applied.

EXPERIMENTAL

TRANSPORT AS A FUNCTION OF TIME: It was shown in the preceding paper (7) that applied 2,4-D rapidly disappears from bean seedlings. While some of the 2,4-D may perhaps be converted to an ether-insoluble complex, no doubt the bulk of this disappearance is due to destruction. Destruction is therefore going on throughout a transport experiment. For this reason the time which can be allowed for transport is limited. Fortunately, the transport is relatively rapid. The general distribution of 2,4-D as a function of time after application is shown in table I. The figures are the actual amounts in micrograms, determined from the calibration curve carried out with pure 2,4-D at the same pH as the extract and on the same day.

It is clear that 2,4-D travels from the point of application (the leaf flap) into other parts of the plant. None, however, was recovered from the base of the hypocotyl or the roots, although less than one microgram may have been present there since this is the limit of sensitivity of the method. In any event this would be less than 0.5 % of the total applied. There is evidently a sharp gradient between the top and bottom halves of the hypocotyl, so that the block to movement is not at the root-stem transition but in the hypocotyl.

After 24 hours the amounts of 2,4-D recoverable from all parts of the plant diminish steadily (table I, also table I and fig 3 of reference 7). The decrease in the leaf could, of course, be ascribed to transport out of it, but the failure of 2,4-D to increase in the stem after 24 hours indicates rather that transport is no longer occurring. This conclusion was tested by making a second application of 50 μgm of 2,4-D via a new flap in the treated leaf at 24 hours after the first application (table II). At the end of the first 24 hours, 18, 14 and 1 μgm were found in the epicotyl, upper and lower halves of the hypocotyl respectively, while at 48 hours these had diminished to 6, 4 and 0 μgm. Hence, after a second application of the same quantity, if the transport mechanism was

TABLE I

RECOVERY OF 2,4-D FROM VARIOUS PARTS OF 4 BEAN SEEDLINGS AT DIFFERENT TIMES AFTER APPLICATION

TIME IN HRS	RECOVERY OF 2,4-D, μGM						% TOTAL RECOVERY
	VIAL AND FLAP	BLADE AND PETIOLE	EPICOTYL	HYPOCOTYL		ROOTS	
				UPPER HALF	LOWER HALF		
6	150	34	4	0	0	0	94
12	56	90	19	Trace	0	0	82
24	50	56	37	5	0	0	74
36	48	29	33	Trace	0	0	55
48	42	19	27	0	0	0	44

In each case 50 μgm 2,4-D in 0.2 ml water were applied to the primary leaf (total 200 μgm) and uptake was complete in about 15 hrs.

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TABLE II

RECOVERY OF 2,4-D FROM STEMS OF 4 BEAN SEEDLINGS AFTER ONE AND TWO APPLICATIONS TO THE PRIMARY LEAF

STEM PARTS	RECOVERY OF 2,4-D, μGM		
	AFTER 1 APPLICATION		AFTER 2 APPLICATIONS
	24 HRS	48 HRS	48 HRS
Epicotyl	18	6	11
Hypocotyl			
Upper half	14	4	5
Lower half	1	0	0

In each case 50 μgm 2,4-D were applied; second application was made to a new flap 24 hrs after the first. Analyses for recovery of 2,4-D were made at 24 hrs and 48 hrs.

unimpaired, one would expect to find 18 μgm in the top part of the hypocotyl at 48 hours, i.e., 4 μgm left from the original treatment plus 14 μgm from the second application made 24 hours before extraction. As shown in table II, only a third of the expected amount was found, so that little or none of the second application was translocated into the hypocotyl under these conditions. Similarly, in the epicotyl, if transport continued at the initial rate, 18 μgm would be expected to move there in the second 24 hours whereas only 5 μgm appeared. Evidently, therefore, after 2,4-D has been present in the tissue for 24 hours, transport of the 2,4-D is very greatly diminished.

TRANSLOCATION OF 2,4-D AS A FUNCTION OF AMOUNT APPLIED: A series of experiments was conducted in which the dosage of 2,4-D was varied from 12.5 up to 100 μgm per plant. All plants were harvested at 24 hours after application. The actual amounts and the percentage recovered from the different parts are recorded in table III. When only 12.5 μgm of 2,4-D were applied per plant less than 1 % was detected in the stem at 24 hours. As the dosage was raised to 75 μgm per plant the absolute amount transported into the stem increased, although the percentage of the total applied did not increase appreciably. When the dose was further increased to 100 μgm per plant, there was a decrease in the amount transported into both epicotyl and hypocotyl. The amount entering the leaf, however, increased in absolute amount and in percentage, showing the effect of 2,4-D in damaging the transport mechanism is exerted mainly in the stem.

It will be noticed also that the amount destroyed, when 25 μgm or more are applied, is essentially constant. The system or systems which lead to the destruction, or other mode of disappearance, are evidently saturated at this concentration.

NATURE OF THE TISSUES TRANSPORTING 2,4-D: In a series of experiments, 50 μgm of 2,4-D were applied as usual to one primary leaf of each of 4 plants, and after 24 hours the xylem was separated from the phloem and cortex of the epicotyls and extracted separately. In the first trial, the phloem and cortex contained 11 μgm and the xylem 13 μgm; in another trial using only part of the epicotyl, 5 μgm were recovered from the phloem and cortex and 6 from the xylem. Thus, the 2,4-D was almost equally divided between the two regions. This suggests that all tissues may participate in transporting 2,4-D. Experiments with ringed plants, however, disprove this possibility. In the intact plant, 14 μgm of 2,4-D were recovered in the upper half of the hypocotyl. If, however, a ring of cortex and phloem 1 cm long was removed from the bottom of the epicotyl, movement into the hypocotyl was completely stopped (table IV, second column). Downward movement, therefore, took place in the region of the phloem and cortex, or possibly in the region of the cambium and its young derivatives, which is largely destroyed in ringing operations. It may be deduced that the 2,4-D found in the xylem of the epicotyl probably moved there laterally from the adjacent cortex. This general behavior agrees with the earlier findings of Skoog (17) on the movement of IAA in tomato plants.

ROLE OF CARBOHYDRATES IN THE TRANSLOCATION OF 2,4-D FROM THE LEAF: Some of the earliest experiments with 2,4-D (11, 13) suggested that it was not translocated in the dark. When bean seedlings were kept in the dark before and after the application of 2,4-D to the primary leaf, the growth response of the epicotyl was not observed. If, however, sugar was applied to the leaves with the 2,4-D the epicotyl did curve (15, 20). Unfortunately the result is inconclusive, for while the sugar may indeed have brought about the translocation of the auxin, it could equally well have exerted its effect by making it possible for the growth response to be exhibited.

The data presented in table V show directly that the 2,4-D is not transported from bean leaves in the dark. These plants were kept in the dark for 18 hours prior to the application of 50 μgm of 2,4-D and for 24 hours thereafter. In the dark, the 2,4-D was taken up and actually moved from the flap to the remainder of the leaf, but movement into or down the

TABLE III

RECOVERY OF 2,4-D FROM VARIOUS PARTS OF BEAN SEEDLINGS AT 24 HRS WHEN DIFFERENT AMOUNTS WERE APPLIED TO THE PRIMARY LEAF IN 0.2 ML OF SOLUTION PER PLANT

AMT 2,4-D APPLIED TO 4 PLANTS	RECOVERY OF 2,4-D				TOTAL RECOVERY	AMT DESTROYED
	LEAF	EPICOTYL	HYPOCOTYL			
			UPPER HALF	LOWER HALF		
μgm	μgm	μgm	μgm	μgm	%	μgm
50	22	1	0	0	46	27
100	39	8	2	0	49	51
200	100	33	4	0	69	63
300	190	61	9	0	86	40
400	294	45	1	0	86	60

TABLE IV

RECOVERY OF 2,4-D FROM VARIOUS PARTS OF 4 BEAN SEEDLINGS 24 HRS AFTER 50 μGM (TOTAL 200 μGM) WERE APPLIED TO THE PRIMARY LEAF UNDER CONDITIONS WHICH MODIFY TRANSLOCATION

CONDITIONS	RECOVERY OF 2,4-D, μGM					
	VIAL AND FLAP	BLADE AND PETIOLE	EPICOTYL	HYPOCOTYL		ROOTS
				UPPER HALF	LOWER HALF	
Intact plants	58	29	18	14	1	0
"Ring" at base of epicotyl	64	58	12*	0	0	..
Roots in 0.12 M mannitol solution	68	14	14	6	0	0

* Includes ringed area.

stem was negligible. As a result, large quantities of 2,4-D accumulated in the leaf. When 10% sucrose was painted on the leaves at the same time as the 2,4-D was applied in the dark, movement out of the leaf did occur. This movement was not as extensive as that occurring in the light, since more was found in the upper half of the hypocotyl under the latter conditions. However, the evidence does confirm the conclusion (13, 15, 20) that light, or the carbohydrate formed thereby, is essential for the transport of auxin from the leaves.

Table V also shows that addition of sucrose in the light did not enhance the transport of 2,4-D nor did the addition of sucrose some hours after the application of the auxin increase the movement. For example, when 10% sucrose was painted on the leaves at 9 hours, 36 and 3 μgm were recovered from the epicotyl and top of the hypocotyl respectively, while 48 and 9 μgm were recovered from these parts of plants which received the same amount of 2,4-D but no sugar. Thus, while carbohydrate can substitute for light to make transport from carbohydrate-deficient leaves possible, it does not add to the effect of light.

Many workers (2, 3, 4, 9, 10, 11, 13, 20) have interpreted the influence of carbohydrate on 2,4-D transport in terms of the mass flow hypothesis of Münch (12), believing that the 2,4-D is swept along with a mass flow out of leaves. There is, in general, a deficiency of strong evidence to support the mass flow hypothesis, and in this particular case it is especially weak because the movement of 2,4-D in the dark has been induced only with carbohydrates. These are undoubtedly active osmotically, but on the other hand they might also be acting as a source of energy for a metabolic process. In other words, the role of sucrose, glucose and fructose in facilitating 2,4-D transport could be that of providing energy for an active transport mechanism. In the tentacles of Drosera both uptake and transport of various organic compounds are active processes (1). Transport of auxin in the Avena coleoptile is clearly an active process also (19). Went (21) has drawn a parallel between auxin transport and the active accumulation of ions against gradients as exhibited by roots and algae.

An attempt was made to increase the gradient of suction force between leaves and roots by removing water from the roots. This was done by substituting the tap water of the flasks with a 0.12 M mannitol solution. As shown in the last column of table IV the 2,4-D did not move into the roots or the lower parts of the hypocotyl under these conditions.

Table VI shows the results of attempts made to induce the transport of 2,4-D in the dark by applying compounds other than sucrose to the leaves. These compounds, urea, mannitol and arabinose, were chosen because, while osmotically active, they probably could not act as sources of energy in starch-depleted leaves. In the dark 0.29 M or 0.15 M sucrose could bring about as much translocation of 2,4-D into the epicotyl as in light (cf table V). However, neither 0.1 M nor 0.15 M mannitol could duplicate this activity. Unfortunately, the mannitol quite evidently inhibits uptake of 2,4-D into the leaf-blade and petiole. Higher concentrations of mannitol were not used because, unlike sucrose, they caused slight wilting of the edges of the leaf. This wilting indicates that the mannitol is not readily taken up hence, perhaps, not enough was entering the leaf to activate a mass flow. Nevertheless, arabinose, even at 0.2 M, could not facilitate the transport of 2,4-D from the leaves (table V). Urea is known to enter cells readily causing reversal of plasmolysis, and to be metabolized as a nitrogen source. Solutions of urea at 0.15 M and 0.25 M, neither of which appeared to affect the turgidity of

TABLE V

EFFECT OF SUCROSE IN LIGHT AND DARK ON RECOVERY OF 2,4-D FROM VARIOUS PARTS OF 4 BEAN SEEDLINGS AFTER 24 HRS

PARTS MEASURED	RECOVERY OF 2,4-D, μGM			
	IN THE LIGHT		IN THE DARK *	
	CONTROL	SUCROSE	CONTROL	SUCROSE
Vial	15	0	27	30
Flap	43	30	26	28
Blade	20	17	103	37
Petiole	9	3	7	18
Epicotyl	18	17	1	20
Hypocotyl				
Upper half	14	19	0	1
Lower half	1	1	0	0
Total μgm	120	87	164	134
%	60	44	82	67

Fifty μgm 2,4-D were applied to the primary leaf (total 200 μgm) with and without the addition of 10% sucrose solution.

* Plants were kept in the dark for 18 hours before application.

the leaves, were totally unable to activate transport of 2,4-D. Table VI shows that there was no inhibition of uptake. Thus, none of the compounds tested is capable of duplicating the action of sucrose under these conditions. This agrees with recent results of Jaworski et al (10) which have appeared since this work was done. These workers tested the ability of a number of compounds to induce the transport of radioactive 2,4-D from the leaves of etiolated bean seedlings. Sucrose and glucose brought about big increases in the amount of C^{14} in the stems but other compounds did not.

EFFECT OF LOW TEMPERATURE ON TRANSPORT OF 2,4-D: The occurrence of a mass flow movement could be tested in another way. If sucrose and 2,4-D were moving simultaneously in response to a differential in osmotic pressure, the transport should be relatively insensitive to temperature changes. It has been claimed by Hauser and Young (5) that there is no transport of 2,4-D in the light at low temperatures. However, the response in their tests depended on growth, which would be negligible at low temperatures, so that a positive test could not have been given by this method even if transport had occurred. Indeed, Kelly (8) has shown that 2,4-D in concentrations high enough to cause drastic growth distortions and toxicity at 30° C had no visible effect at 4° C. Tests depending on growth of the treated plants are therefore unsuitable in this case.

The present method was used to determine the amounts of 2,4-D transported at 19° C and 4° C. All plants were kept in the dark at 19° C for 24 hours after which time 50 μgm of 2,4-D were applied to one primary leaf and the leaf painted with a 0.3 M sucrose solution as above. Some of the plants were kept at 19° C and others at 4° C. Extractions were made 24 hours later. Untreated plants returned to room temperature after being kept at 4° C for 24 hours showed no ill effects from the exposure to cold.

As shown in table VII, translocation out of the leaf at 4° C was negligible, although movement from the flap to the remainder of the leaf occurred readily. It appears, therefore, that movement within the leaf may depend on a temperature insensitive process while movement out of the leaf is temperature dependent.

TABLE VI

EFFECT OF SUCROSE AND OTHER SOLUTES ON RECOVERY OF 2,4-D FROM VARIOUS PARTS OF 4 BEAN SEEDLINGS 24 HRS AFTER APPLICATION IN THE DARK *

PARTS MEASURED	RECOVERY OF 2,4-D, μGM								
	CONTROL	SUCROSE		MANNITOL		ARABINOSE	UREA		
		0.29 M	0.15 M	0.15 M	0.1 M	0.2 M	0.25 M	0.15 M	
Vial and flap	49	58	..	75	71	..	22	..	
Blade and petiole	106	55	..	13	29	..	119	..	
Epicotyl	1	20	11	0	0	1	0	0	
Hypocotyl									
Upper half	0	0	..	0	0	..	0	..	
Lower half	0	0	..	0	0	..	0	..	

Fifty μgm 2,4-D were applied to the primary leaf (total 200 μgm); other solutions were painted on the same leaf.

* Plants were kept in the dark for 18 hrs before application.

TABLE VII

EFFECT OF TEMPERATURE ON RECOVERY OF 2,4-D FROM VARIOUS PARTS OF 4 BEAN SEEDLINGS 24 HRS AFTER APPLICATION IN THE DARK *

PARTS MEASURED	RECOVERY OF 2,4-D, μGM	
	19° C	4° C
Vial and flap	58	60
Blade and petiole	25	120
Epicotyl	20	1
Hypocotyl		
Upper half	1	2
Lower half	0	0

Fifty μgm 2,4-D were applied to the primary leaf (total 200 μgm) and the 0.29 M sucrose was painted on the same leaf at 4 and 19° C.

* Plants were kept in the dark at 19° C for 24 hrs before application.

DISCUSSION

The results of these experiments show definitely that 2,4-D does not move from leaves in the dark. If, however, sucrose is applied at the same time as the 2,4-D, transport occurs. The frequently offered explanation that the sugar would increase the mass flow by raising the suction force of the leaf cells, and that the 2,4-D would then be carried along with the mass flow is made very unlikely by our experiments. Firstly, the failure of sucrose to increase the transport at 4° C is evidence that the movement is not due to a simple osmotic phenomenon. Secondly, the inability of mannitol, urea or arabinose to induce transport of 2,4-D in the dark implies that some special characteristic of sucrose, other than its osmotic property, is concerned. Particularly in the case of urea, which is known to be readily taken up by leaves and to enter cells rapidly, some increase in the suction force must almost certainly occur. Finally, the application of mannitol to the roots does not affect the transport.

There is, of course, considerable evidence in the literature against the mass flow hypothesis, at least, in its simple form. The morphology of sieve-plates themselves would appear to make free flow difficult. Schumacher's evidence (16) shows that the movement of fluorescein in leaf veins is quite independent of changes in turgor pressure brought about by local plasmolysis, or in fact of the leaf parenchyma itself. Roeckl's findings (14) that palisade cells can be plasmolyzed in phloem sap makes it difficult to see how

solutes could pass from leaf parenchyma into phloem by a simple osmotic mechanism. Wanner has shown (18) that leaf cells contain mainly hexoses and their phosphates, while sieve tubes contain only sucrose, which makes it clear that metabolic processes participate at least in the entry of solutes into sieve tubes. If we conclude that the movement of solutes from the palisade cells to the phloem is an active step then the present work is consistent with the view that the transport of 2,4-D or, at least, a step limiting its transport, is a metabolic process. Carbohydrate would be required as a source of energy so that compounds unable to furnish energy would be incapable of promoting transport. Such a step would also be sensitive to decreases in temperature.

After 24 hours, further downward transport came almost to a standstill and the amounts found in the stem readily diminished. Apparently, therefore, 2,4-D disrupts the transport mechanism responsible for its own movement. The reduced transport when large amounts are applied (100 μgm per plant—table III) shows that the disruption can even take place in less than 24 hours. These data also are in accord with the concept of a metabolic process controlling the transport. Studies are reported elsewhere (see table II of reference 6) on the ability of short excised sections taken from different parts of the root and stem of bean seedlings of this age to transport IAA in a polar direction. Sections taken from the root-stem transition zone or from the roots could not transport as much auxin per unit time as could sections taken from the stems. If this mechanism operates in intact plants, a possible block in downward transport of auxin may exist there. On the other hand, all parts of the stem were found to be able to transport IAA in relatively large amounts. There is, therefore, no reason to believe that the lower part of the stem is unable to transport auxins by this mechanism and, hence, the failure to transport 2,4-D through the hypocotyl must be due to another cause.

In this same work (6) it was shown that 2,4-D has a detrimental effect on the mechanism for the transport of IAA in excised sections of bean stems. Stem sections taken from plants which had received a foliage application of 2,4-D 24 hours beforehand almost completely lacked the ability to translocate auxin. A similar effect on the transport mechanism for 2,4-D in the intact plant (whether or not it is the same mechanism) would account for many of the results observed in the present work.

The cessation of 2,4-D transport in the hypocotyl is not easy to explain. Its consequence, namely that 2,4-D is not conducted in appreciable amounts from leaves to roots, is of importance in herbicide work particularly with deep-rooted perennials. Numerous field observations with a variety of plants have pointed in the same direction but the present data are believed to constitute the first direct evidence. The appearance of isotope in roots when 2,4-D containing C^{14} is supplied may doubtless be ascribed to breakdown products.

Summary

2,4-D was applied in aqueous solution to a cut flap of the primary leaf of Phaseolus seedlings growing in water under controlled conditions. Uptake and movement of 2,4-D were then followed by quantitative bioassay of partially purified ether extracts.

The transport, which is accompanied by extensive breakdown, ceases at about the middle of the hypocotyl, none entering the roots at any time. Transport is proportional to the amount applied up to 75 μgm per plant, larger amounts inhibiting the transport out of the leaf. The amount of 2,4-D disappearing in 24 hours, on the other hand, was essentially constant when 25 μgm or more was applied per plant.

The transport was inhibited completely by ringing, although analysis showed 2,4-D about equally distributed between xylem and cortex.

Transport did not take place in the dark (the plants being kept in the dark beforehand) but could be induced by applying sucrose solution to the leaf of the darkened plant. Sucrose did not increase the transport in the light, nor did it exert its effect at 4° C. Neither mannitol, arabinose nor urea could substitute for sucrose. Application of mannitol to the roots also did not facilitate transport downward.

It is deduced that transport of 2,4-D is not due to simple osmotic forces as in the mass flow hypothesis, but involves a metabolic component.

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INDOLEACETIC ACID AND THE UTILIZATION OF RADIOACTIVE PYRUVATE AND ACETATE BY WHEAT ROOTS[1,2]

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Auxins and antiauxins are known to have rather striking effects on the utilization and evolution of acetaldehyde by various plant tissues (10). In concentrations that inhibit growth auxins and antiauxins cause the evolution of acetaldehyde. At lower concentrations (growth promoting in the case of indoleacetic acid) there is an appreciable enhancement of acetaldehyde consumption. The acetaldehyde work encouraged further study with C^{14}-labeled pyruvate and acetate. Some of this work relating to the conversion of acetate-1-C^{14} to various water soluble constituents and lipides of wheat roots has been reported (11). At a concentration of 2.5×10^{5} M, indoleacetic acid (IAA) inhibits the absorption of acetate and synthesis of non-volatile acids. Accumulation of free acetate is markedly enhanced, and synthesis of lipides is increased by as much as 86 % by IAA.

In an earlier report, Boroughs and Bonner (1) describe similar experiments with Avena coleoptiles in which the effects of IAA on the conversion of acetate-1-C^{14} and other labeled compounds to a number of plant substances were studied. Of the groups of compounds analyzed only the synthesis of noncellulosic polysaccharides is impressively affected (stimulated by 46.3 %) by IAA. An enhancement of lipide synthesis by 4 to 10 % was observed and effects on cell wall materials, excluding noncellulosic polysaccharides, range from + 13.5 % for protopectin to − 10.5 % of pectate. No data on the effects of IAA on absorption of the radioisotope are given.

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MATERIALS AND METHODS

Roots excised from etiolated wheat seedlings (White Federation 38) grown for 4 days in distilled water were used in the experiments described here. After excision, the roots were washed, dried by centrifugation and weighed.

Pyruvic acid used in the first experiment of this study was prepared by hydrolysis of pyruvamide-2-C^{14} purchased from Tracerlab, Inc. The amide, having a specific activity of 0.5 mc/mM, was refluxed in a tube for 5 hours with 1.7 ml of 0.4 *N* KOH. This solution was acidified and extracted with ether. Pure pyruvic acid was added to the radioactive acid in the ether and the potassium salt then prepared by addition of K_2CO_3. The salt was precipitated by the addition of dioxane and separated from the solvent by centrifugation. Following complete removal of the solvent, the potassium pyruvate was dissolved in water and used in the experiment. In view of the large losses of radioactivity involved in the purification of the pyruvate we decided to use radioactive acetate in subsequent work. Acetic acid-1-C^{14} having a specific activity of 3.72 mc/mM after repurification by vacuum distillation was obtained from Professor R. F. Nystrom of this University.

For the pyruvate experiment two sets of wheat roots each weighing 22 gm were incubated at 25° C for 6 hours in 250 ml of potassium pyruvate (8×10^{-4} M) adjusted to pH 5.0 with H_2SO_4 and having initially 13.4 μc of radioactivity. Indoleacetic acid at 1×10^{-4} M was present in the mixture supplied to the auxin-treated roots. During the experiment a stream of CO_2-free air passed through the closed vessels containing the roots. The air stream in each case then passed through a U tube held at − 80° C (by a solid CO_2-methyl cellosolve mixture) to retain vola-

tile materials and finally through 2 gas-washing bottles: the first containing 0.1 *N* NaOH; the second containing 0.05 *N* $Ba(OH)_2$ to trap CO_2.

At the end of the experimental period the roots were washed and frozen. External solutions, washings from the roots and from the – 80° C trap were combined. These solutions and aliquots of the original solutions were analyzed for pyruvate (18) for a measure of pyruvate uptake. Sap was expressed from the frozen roots by an hydraulic press. Soluble substances remaining in the press cakes were extracted by the addition of water followed by reapplication of pressure. Six washings with water brought the volume of the press sap to about 100 ml. The press cakes were dried at 60° C.

Combined sap and washings from each sample were passed successively through a cation exchanger (Amberlite 100-H) and an anion exchanger (Amberlite 1R4-B). A mixture containing 10 ml of *N* NH_4OH, 3 ml of *N* NaOH and 37 ml of H_2O was used to elute the anion column. The column was washed with about 100 ml of water and the combined washings and eluate were dried on a steam bath. Separation of the acids in the eluate was effected on silica gel by the method of Bulen et al (2). Malic acid isolated from each sample was diluted with an equal amount of non-radioactive malic acid and the diphenacyl esters prepared by a method described by Shriner and Fuson (16). Samples of the malic esters were assayed for radioactivity by a dry combustion technique in which the sample is burned to CO_2 in a micro-Pregl combustion apparatus, the CO_2 enters a high vacuum line where it is held in a – 180° C trap while other gases are removed, the volume of the CO_2 is then measured manometrically and finally the CO_2 is transferred to an evacuated ionization chamber for measurement of radioactivity. Activity in the chamber is measured on a vibrating reed electrometer (14). The carbon content of the sample serves as proof of its identity.

The deionized sap was treated by the method of Noggle and Balomey (13) for the quantitative recovery of free sugars. Glucosazones were prepared from the sugars by a method outlined in Shriner and Fuson (16). They were assayed for radioactivity by the dry combustion technique.

Carbon dioxide in the alkali traps was converted to $BaCO_3$ by the method of Calvin et al (4). Samples of the $BaCO_3$ were treated with perchloric acid on the vacuum line, and the CO_2, after its volume had been determined, was transferred to the ionization chamber for assay.

The dried press-cakes, weighing 944.2 mg and 1109.3 mg from the control and IAA-treated samples respectively, were each triturated with 15 ml of 17.5 % NaOH. After 48 hours in the alkaline solution the remaining solid material was separated from the dissolved materials on tared, sintered glass filters. The solids were washed with water until they were free of alkali, then dried and weighed. Residual material on the filters consisting primarily of α-cellulose amounted to 424.6 mg for the control and 443.7 mg for the IAA-treated tissues respectively. Each of these samples was covered with 4 ml of 72 % H_2SO_4, and allowed to stand 6 days. Then the digests were filtered and the residue washed thoroughly with water. With an allowance for the undissolved material in the filters it can be calculated that the sulfuric acid solutions contained 396.6 mg and 435.0 mg of glucose in the control and auxin-treated samples respectively. Aliquots of the sulfuric acid solutions were assayed for radioactivity by a wet oxidation procedure (12). In this method the organic material is oxidized by chromic acid (19), and the gaseous products are carried to an evacuated ionization chamber by a stream of CO_2. As in the other technique, activity in the chamber is measured on a vibrating reed electrometer.

Cell wall materials in the alkaline extracts (chiefly pectic substances, polyuronide hemicelluloses, and non-cellulosic polysaccharides), determined by difference, amounted to 519.8 mg for the control and 604.6 mg for the auxin-treated samples. Aliquots of the alkaline solutions were assayed for activity by the wet oxidation procedure.

For the 4-hour acetate experiment 27 gm of roots were incubated at 25° C in 100 ml of 1.5×10^{-4} M acetate solution adjusted to pH 4.7 with KOH and having 372 μc of activity. Indoleacetic acid at 1×10^{-4} M was present in the mixture supplied to the auxin-treated roots. As in the pyruvate experiment the roots were aerated with CO_2-free air and the – 80° C trap and the alkali traps were included.

At the end of the experiment the roots were removed from the external solutions and washed thoroughly. External solutions, washings from the roots and the cold traps were combined and assayed for activity along with aliquots of the original solutions to obtain a measure of C^{14} taken up. All assays for radioactivity in this experiment were by the wet oxidation procedure. The roots were frozen, then blended two successive times in ice water. These suspensions were filtered through sintered glass and washed with ice water. The aqueous extracts of the tissues were assayed for total activity.

The insoluble materials were fractionated for various cell wall constituents. Removal of the pectic substances was effected by our extracting the solids 4 times with 0.05 *N* HCl at 80° C in a Wye-Phillips extractor (8). After the cell wall material was washed an additional time with water the combined extracts and washings were adjusted to pH 7.0, the volumes reduced to 50 ml and aliquots taken for assay.

Solids remaining from the acid extraction were homogenized in water and aliquots taken for C^{14} analysis. This fraction is designated "holocellulose minus pectic substances." The bulk of this material was dried then triturated with 4 % NaOH solution and left in contact with the alkali for 48 hours. Dissolved materials were filtered off the solid residue and latter washed. Aliquots of the 4 % NaOH-ex-

tract, hemicelluloses by definition, were assayed for activity.

The solid residues were dried again and extracted with 17.5 % NaOH for 48 hours. Soluble materials, non-cellulosic polysaccharides, were separated from the solid material by filtration on sintered glass. Aliquots of the alkaline extracts were assayed. Undissolved material, largely α-cellulose, was suspended in water and aliquots analyzed for radioactivity. Aliquots of the solutions in the CO_2 traps were also analyzed for activity.

For the 2-hour acetate experiment, 19 gm of wheat roots were incubated at 21.4° C in 100 ml of 6×10^{-4} M acetate solution adjusted to pH 4.7 with KOH and having activities of 237.65 μc in the control and 224.42 μc in the auxin treatment. The latter included IAA at 2.5×10^{-5} M. As in the previous experiments, the roots were aerated, and traps for volatile materials and CO_2 were included. Fractionation of the plant material followed the procedures used in the previous acetate experiment.

Results

Absorption of the labeled pyruvate and acetate is significantly inhibited by IAA. When the relative incorporation of activity into various cellular constituents by the auxin-treated and control tissues is calculated on the basis of equal absorption of the isotope it becomes apparent that the distribution of radioactivity within the tissues is also influenced by IAA.

IAA inhibits synthesis of malic acid and the soluble sugars from pyruvate (table I). The aqueous extract from the auxin-treated roots in the 4-hour acetate experiment has a greater activity than the extract from the control roots (table II). This probably reflects an effect reported earlier (11), the accumulation of free acetic acid in roots supplied with IAA and radioactive acetate.

Of the cell wall materials the synthesis of polyuronide hemicelluloses appears to be most profoundly restricted by IAA (tables II and III). Unfortunately, separate analyses for pectic materials, hemicelluloses and non-cellulosic polysaccharides were not made in the pyruvate experiments. These constituents undoubtedly make up the bulk of the material dissolved in the 17.5 % NaOH extract of the cell wall substances of the pyruvate experiment. Synthesis of these groups of compounds taken together is appreciably reduced by IAA (table I).

Total activity of the pectic substances exceeds that of any other group of cell wall materials. A significant inhibition is produced by IAA at 1×10^{-4} M in the 4-hour experiment but in the shorter time period the effect is not impressive. Little activity is incorporated into the non-cellulosic polysaccharides. Synthesis of these groups of substances is appreciably reduced by IAA (table I).

It is interesting to find that the synthesis of one cell wall constituent, α-cellulose, is consistently stimulated by IAA (tables I, II, and III). Results of the 2-hour experiment pertaining to activities in the non-volatile acids and acetic acid can be found in the

Table I

Effects of IAA on the Utilization of Pyruvate-2-C^{14} by Wheat Roots

	Control	IAA	IAA/control % *
Pyruvate absorbed, mg	1.88	1.25	100
Malic acid, μc/millimole	8.07	3.02	58
Soluble sugars, μc/millimole	2.61	1.28	74
α-Cellulose, μc/mg	1.21	1.47	183
Other cell wall materials, μc/mg	4.25	2.98	64
Respiratory CO_2, μc/millimole	5.55	2.25	61

Twenty-five gm of roots were incubated at 25° C for 6 hrs in 250 ml of 8×10^{-4} M pyruvate solution adjusted to pH 5.0 with KOH and having initially 13.2 μc of activity. The IAA concentration was 1×10^{-4} M.

* Calculated on the basis of equal uptake of pyruvate.

Table II

Effects of IAA on the Utilization of Acetate-1-C^{14} by Wheat Roots in 4 Hrs

	Total activity, μc		IAA/control % *
	Control	IAA	
C^{14} absorbed	287.1	225.1	100
Aqueous extract	93.7	98.3	131
Pectic substances	22.2	14.3	82
Hollocellulose minus pectic subst.	8.05	4.78	76
Polyuronide hemicelluloses	5.76	2.92	65
Non-cellulosic polysaccharides	0.24	0.13	69
α-Cellulose	1.35	1.22	115
Respiratory CO_2	99.00	74.5	95

Roots weighing 27 gm were incubated at 25° C for 4 hrs in 100 ml of 1.5×10^{-4} M acetate solution adjusted to pH 4.7 with KOH and having 372 μc of activity in each treatment. The IAA concentration was 1×10^{-4} M.

* Calculated on the basis of equal uptake of C^{14}.

Table III

Effects of IAA on the Utilization of Acetate-1-C^{14} by Wheat Roots in 2 Hrs

	Total activity in μc		IAA/control % *
	Control	IAA	
C^{14} absorbed	214.40	178.97	100
Aqueous extract	121.00	129.50	129
Pectic substances	11.91	9.49	96
Hollocellulose minus pectic subst.	2.89	2.07	85
Polyuronide hemicelluloses	2.38	1.47	74
Non-cellulosic polysaccharides	0.06	0.05	99
α-Cellulose	0.50	0.48	115
Respiratory CO_2	15.70	13.96	101

Roots weighing 19 gm were incubated at 25° C for 2 hrs in 100 ml of 1×10^{-4} M acetate solution adjusted to pH 4.7 with KOH and having 237.65 μc in the control and 224.42 μc in the auxin-treatment. The IAA concentration was 2.5×10^{-5} M.

* Calculated on the basis of equal uptake of C^{14}.

earlier publication (11). Previously published data on absorption of acetate, and activities in the aqueous extract and acetic acid are included in table III for convenience.

Discussion

It appears that two categories of auxin effects can be distinguished in these experiments and the ones reported earlier (11). One group of responses to IAA clearly related to processes directly concerned with acetate metabolism includes inhibited uptake of acetate and pyruvate, inhibited syntheses of malic and other non-volatile acids, accumulation of free acetic acid, and enhanced synthesis of lipids. To the other group of auxin effects, none of which appears to be directly concerned with acetate metabolism, one can assign inhibited syntheses of polyuronide hemicelluloses, pectic substances and free sugars, and enhanced formation of α-cellulose. In the pyruvate experiment and in an acetate experiment of 30 minutes duration (11), IAA inhibited formation of $C^{14}O_2$ to about the same degree that it inhibited incorporation of activity in the organic acids. In the 2-hr and 4-hr experiments no significant effect on the production of $C^{14}O_2$ is observed. Formation of $C^{14}O_2$ is apparently not solely affected by the incorporation of activity into acids of the Krebs cycle.

It may be argued that the significance of these experiments is limited because the treatment with IAA is an inhibitory one. Studies involving the promotion of growth by antiauxins for example may prove more interesting. It is true, however, that these experiments permit a comparison of the activities of growing roots (the controls) with non-growing roots (the IAA-treated tissues). It seems significant that synthesis of material characteristic of the relatively plastic primary cell wall, polyuronide hemicelluloses and pectic substances, is greater in the growing roots. In the non-growing roots greater synthesis of a material characteristic of the rigid, secondary cell wall, α-cellulose, occurs. Some of this new cellulose is probably in the primary cell wall and may not necessarily add to the rigidity of the cell wall. Kerr (7) is of the opinion that the cellulose fibers of the primary cell wall are disconnected and dispersed in a continuous matrix of pectic substances. If the primary cell wall of wheat roots is put together in this fashion one would expect additional synthesis of cellulose at this site might have little or no effect on wall plasticity. In view of the evidence in Burström's work (3) indicating that growth in wheat roots involves an increase in the plasticity of the cell wall, it seems reasonable to assume that inhibition of growth is associated with an increase in wall rigidity which is due in part at least to the deposition of α-cellulose.

It is possible that the changes in cell wall composition are a consequence of an auxin effect involving acetate or enzymes and coenzymes concerned with acetate. There is no direct proof of such a relationship in these experiments but evidence presented by Siegel and Galston (17) for the cleavage of an indoleacetic acid–protein complex by coenzyme A adds to the attractiveness of the idea. Fawcett et al (5) have demonstrated β oxidation of the fatty acid side chains of various phenoxy acid derivatives. This oxidation may involve formation of a coenzyme A derivative of the auxin. This possibility must be considered highly speculative, however, in view of the studies of Humphreys et al (6) on the oxidation of palmitic acid by a preparation from peanuts which does not require coenzyme A. Price and Leopold (15) have recently reported their failure to repeat earlier work by Leopold and Guernsey (9) involving various auxins and coenzyme A.

Summary

Absorption of C^{14}-labeled pyruvate and acetate by wheat roots is inhibited by concentrations of IAA that inhibit root growth. When allowance is made for the effect on uptake of the radioactive material, it is apparent that the distribution of activity within the tissue is also appreciably altered by IAA. Incorporation of activity from pyruvate-2-C^{14} into malic acid, soluble sugars, cell wall materials soluble in 17.5% NaOH, and CO_2 is inhibited by IAA. Synthesis of α-cellulose is enhanced by the auxin. Formation of pectic substances and polyuronide hemicelluloses from acetate-1-C^{14} is inhibited while synthesis of α-cellulose is stimulated by IAA at the two concentrations employed (1×10^{-4} and 2.5×10^{-5} M). With the higher concentration of IAA, incorporation of activity into non-cellulosic polysaccharides and CO_2 is restricted.

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THE CAPACITY OF LEAVES OF BRYOPHYLLUM CALYCINUM TO RECOVER FROM PROLONGED EXPOSURE TO DARKNESS OR TO LIGHT[1]

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Emphasis was placed in a recent paper (1) on the observation that the normal diurnal rhythm of the changes in the starch and organic acid content of leaves of *Bryophyllum calycinum* appears to be closely adapted to the average length of a day or night. If the period of darkness or of light is artificially prolonged, the changes in composition, which had been initiated at their usual speed, come more or less to a standstill after the elapse of about 12 hours. The effects of reactions other than those that are expressed by the major changes in composition can then in certain instances be perceived. Such reactions may not be abnormal, but in the usual course of events their effects are concealed by the larger and more rapid changes of composition, and, if they are allowed to proceed for an unusual length of time, there is a possibility that some degree of damage to the system may occur. It was, accordingly, of interest to expose Bryophyllum leaves to a prolonged period of darkness or of light, and to examine the extent to which the normal variations in composition are resumed after transfer to the other condition of illumination.

In discussing the results, it will be convenient to employ the concept of physiological stress. Disregarding the fact that excision of a Bryophyllum leaf from the plant immediately places it under certain not clearly defined but real stresses, the effects of which eventually become evident from the formation of rootlets at the margins of the leaf, observation shows that such leaves, if picked at sunrise and placed in light, behave in the normal fashion, for malic acid diminishes and starch is formed. Furthermore, it has been demonstrated that excised leaves, when exposed to the usual diurnal alternation of light and dark in a greenhouse, undergo profound changes in composition in response to this alternation for at least 3 days (2). Excision from the plant thus by itself places no serious restriction upon the speed or amplitude of the normal chemical changes.

However, if leaves are exposed to artificially maintained illumination for many hours, increasing evidences of abnormality become apparent (3). They become flaccid, and, after the main chemical changes have been completed, irregular although small oscillations of both starch and organic acid content occur. In the converse case, when leaves are picked at nightfall and placed in darkness at ordinary temperatures, starch diminishes in amount and malic acid is formed for about 12 hours; subsequently the starch remains approximately constant while malic acid slowly decreases. Accordingly, under either condition, the leaves may be assumed gradually to have passed into a stage characterized by an increasing degree of physiological stress.

In the present experiments, individual samples of leaves have been removed from such presumably stressed conditions and have been either illuminated or placed in darkness, as the case may be, for 11 to 14 hours to see to what extent recovery could occur. The main criteria employed were the capacity to resume the normal changes in composition with respect to starch and organic acids.

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Experimental Procedure

The ten samples used for the culture experiment in darkness were collected by the statistical method (4) in the early evening of June 20, 1955, a hot, humid and sunny day. The plants had been grown in soil in crocks in the greenhouse, and the top three pairs of fully developed but still young five-leaflet leaves were used. The leaf-culture troughs were set up in a room maintained at 24° C and 50 % relative humidity, and the room was kept in complete darkness after the leaflets had been arranged with their bases in water, 8:00 P.M. being taken as the zero hour of the experiment. After the expiration of 36, 60 and 84 hours (i.e., at 8:00 A.M. on three successive days), an individual sample was transferred to the greenhouse and placed in a trough exposed to full daylight for 11 hours. The sample was then dried for analysis. Meanwhile, samples had been taken from the troughs in the dark room at intervals chosen so as to afford maximal information regarding the rates of the reactions which affect the main components of the leaves. The precise times at which samples were so withdrawn are indicated in the figures.

The ten samples used for the culture experiment in light were collected in the same way, starting at 5:00 A.M. July 21, 1953 (sunrise 5:36 A.M. daylight saving time), also from plants grown in soil in the greenhouse. The culture troughs were arranged in an insulated room under a bank of fluorescent lights which gave a light intensity of approximately 1100 ft-c at the surfaces of the leaves. The temperature varied between 20 and 22° C and the humidity between 58 and 66 % with the operation of the control equipment which automatically came into play at intervals of from 1 to 2 hours. Zero hour was taken at 6:00 A.M. when the initial control sample was placed in the drying oven. After the expiration of 27, 50 and 74 hours, individual samples were transferred to troughs in the dark room where they were held, respectively, for 11, 12 and 14 hours at 24° C and 50 % relative humidity. Samples were also removed from the culture troughs in light and dried for analysis at times indicated in the figures. Fuller details of the technique and of the treatment of the samples in preparation for chemical analysis have been given in previous papers (1, 5). The analytical methods are described in recent bulletins from this station (6, 7).

Experimental Results

Table I shows the evidence for the uniformity of composition within each set of samples. The fresh weights of the individual samples had a coefficient of variation of 1.2 % in one set and 1.15 % in the other, and the nitrogen and ash contents of both sets were satisfactorily constant. Alkalinity of ash was determined in only a few of the samples of the set cultured in darkness, and only the average value is given since the variability of this quantity in other sets of samples has always been found to be less than that of the total nitrogen. The coefficient of variation of the protein nitrogen in both sets of samples was notably higher than that of the components which would be assumed to remain constant, and plots of the data indicated that a slow but continuous loss occurred. The significance of this observation will be enlarged upon later.

The main analytical results are plotted in figures 1 and 2. The solid lines show the changes in composition in continuous darkness at the left, and in continuous light at the right of the figures. The curves which represent the changes in the amounts of each component are placed side by side, and the data confirm in remarkably close detail the results of previous studies of this species (3, 5).

The effect of the transfer of samples from darkness to light or from light to darkness at successive intervals during the course of the experiment is shown by the broken lines, and it is obvious that with few exceptions the change in composition took place in the direction which would be predicted from earlier results. Whether or not the changes actually followed straight line functions, as they are represented to have done, must be left for subsequent study. The mean rates of the reactions which occurred are indicated by the slopes of these broken lines, and close examination shows that these slopes changed progressively with time. Accordingly, the rates at which the sev-

Table I

Analytical Data on Two Sets of Ten Samples Each of Leaflets of Bryophyllum calycinum Collected by the Statistical Method

Analytical determination	Cultured in light with transfer to darkness (Samples 1953-B)		Cultured in darkness with transfer to light (Samples 1955-C)	
	Mean ± S.D.*	C.V., %*	Mean ± S.D.*	C.V., %*
Initial fresh wt., gm/sample	311.6 ± 3.86	1.2	176.2 ± 2.03	1.15
Total N, gm**	1.95 ± 0.033	1.7	2.51 ± 0.031	1.3
Protein N, gm**	1.51 ± 0.040	2.6	1.93 ± 0.050	2.8
Ash, gm**	11.86 ± 0.12	1.02	9.78 ± 0.15	1.6
Alkalinity of ash, meq**	286.2 ± 1.7	0.6	245.3	...

* S.D., standard deviation; C.V., coefficient of variation as percentage.
** Data expressed in terms of 1 kilo of initial fresh wt.

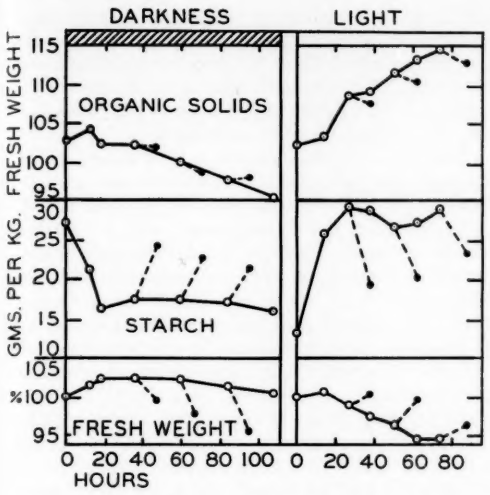

FIG. 1. Changes in organic solids, starch and in fresh weight of leaves of *Bryophyllum calycinum* cultured in water. The solid lines show at the left the effect of culture in darkness (samples 1955-C), those at the right the effect of culture in light (samples 1953-B). The broken lines show the effect of transfer of samples from culture in darkness to light at the left, and of transfer from light to darkness at the right.

eral reactions occurred after transfer are dependent upon the time that had elapsed before the transfer was made.

Before considering this aspect of the data, it is necessary to draw attention to certain details. The curve for organic solids in figure 1 shows that a small increase occurred during the first 12 hours in darkness as would be expected from previous work (1, 5), but that this was followed by continuous loss. Transfer to light, however, gave rise to no marked increase in organic solids; during 11 hours, the sample transferred at the 36-hour point maintained its weight, that at 60 hours lost 1.5 gm per kilo, and that at 84 hours gained only 0.25 gm per kilo. Nevertheless, these 3 samples increased in starch content by 6.5, 4.9 and 4.0 gm per kilo in the 11 hours each of them was exposed to light. Photosynthesis (i.e., net fixation of carbon dioxide) is thus entirely inadequate to account for the formation of the starch. On the other hand, the samples cultured in light increased continuously in organic solids as would be expected. Transfer to darkness brought about losses of organic solids within the next 11 to 14 hours of 0.9, 1.3 and 1.7 gm after, respectively, 27, 50 and 74 hours of culture in light. It would seem that, under the conditions of stress occasioned by long exposure to light, the capacity of the leaves to continue to accumulate organic solids for a short time in darkness is seriously interfered with, although more detailed examination will be required to discover whether or not it is eliminated.

Attention should also be directed to the changes in fresh weight in figure 1. The leaves placed in darkness quickly took up about 2% of water and became unusually turgid; even the sample held in darkness for 108 hours was still crisp although the weight had dropped almost to its original value and evidence of the initiation of rootlets at the margins of the leaves was noted. The samples transferred to light, however, soon lost their turgidity and were definitely limp at the end of the period of exposure.

The samples cultured in light increased slightly in fresh weight for the first 14 hours, but subsequently lost weight continuously and became markedly flaccid; nevertheless, on transfer to darkness, they retained a moderate capacity to take up water again although only the sample exposed to light for 27 hours recovered all of its initial fresh weight.

The behavior of the starch is also shown in figure 1. The two curves are obviously symmetrical with each other in general pattern, and it is unfortunate that the samples were not removed at precisely the

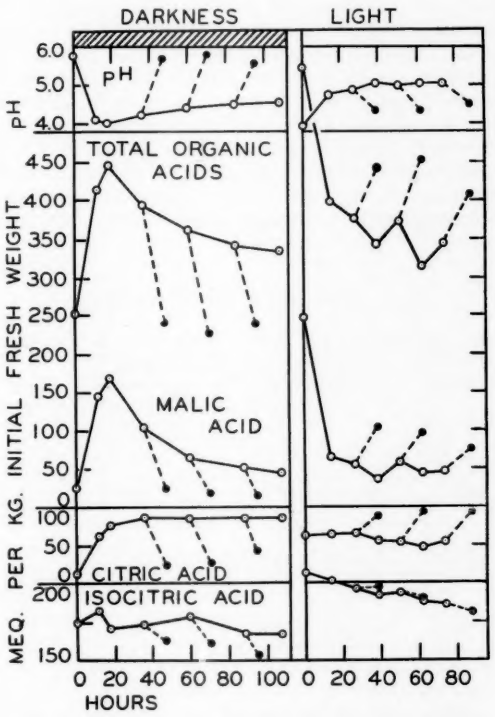

FIG. 2. Changes in pH and organic acids in leaves of *Bryophyllum calycinum* cultured in water. The solid lines show at the left the effect of culture in darkness (samples 1955-C), those at the right the effect of culture in light (samples 1953-B). The broken lines show the effect of transfer of samples from culture in darkness to light at the left, and of transfer from light to darkness at the right. The total organic acids of the sample collected at 0 hours in the set exposed to light was 572 meq per kilo of initial fresh weight, the isocitric acid content of the same sample was 207 meq.

same time intervals so that a calculation of the coefficient of correlation would be justified. The symmetry suggests, however, that the behavior of the two sets of samples was reasonably comparable even though the leaves were subjected to experiment two years apart in point of time.

Data on the rates at which starch disappears in darkness or is synthesized in light in a number of experiments with Bryophyllum leaves are assembled in table II. The sets of samples labeled 1955-C and 1953-B are those for which the full information is shown in figures 1 and 2; more complete data for 1952-A, 1951-C and 1951-B are to be found in references (1, 5, 3), but the data for 1953-A have not been published. The rate of change of starch is expressed in millimoles per hour per kilo of initial fresh weight ($C_6H_{10}O_5$ is taken as 1 mole), and the data give the mean rates during the total indicated periods of exposure. Thus the leaves of set 1955-C in the unstressed condition, that is, immediately after collection in the evening, lost starch at the mean rate of 3.2 millimoles per hour during the first 12 hours and at 3.7 millimoles per hour during the first 18 hours in darkness. It will be noted that the curve in figure 1 slopes downward a little more steeply in the interval between 12 and 18 hours, and thus the mean rate for the whole 18-hour period is slightly increased. Consideration of the figures in column 4 of table II shows that the rate at which starch disappears from Bryophyllum leaves in darkness, provided that they are unstressed or have been stressed for not more than 27 hours of exposure to light, is moderately constant. The figures range from −3.2 to −5.8 millimoles per hour. Within these limits, therefore, different sets of samples of leaves from the clone grown in this laboratory are comparable with each other.

The rates at which starch is synthesized in Bryophyllum leaves collected at sunrise and placed in light are shown in the last column of table II. The range observed for the different sets of unstressed samples is from +5.6 to +12 millimoles per hour for periods of from 10 to 18 hours. Samples 1952-A appear to have been unusually vigorous in this respect. The rates observed in samples that had been stressed by exposure to darkness for 24 or more hours are, however, all smaller and suggest that such exposure interferes to a greater or less extent with the subsequent capacity of the starch-synthesizing mechanism.

The behavior of the organic acids of samples 1955-C and 1953-B is shown in figure 2, and it is obvious that the transformations which led to the formation or loss of malic and citric acids played the largest part in the observed changes in pH and in total organic acids. The behavior of malic acid during prolonged culture of the leaves in darkness is complex since two main effects are superposed; there was a rapid synthesis during the first 18 hours, but malic acid subsequently diminished at a rate such that, after 108 hours in darkness, the level had dropped almost to that in the freshly picked leaves.

TABLE II

RATE OF CHANGE OF THE STARCH CONTENT OF LEAVES OF BRYOPHYLLUM CALYCINUM DURING EXPOSURE TO DARKNESS OR TO LIGHT

SAMPLES	CONDITION BEFORE TREATMENT	TOTAL EXPOSURE TO DARKNESS	RATE	CONDITION BEFORE TREATMENT	TOTAL EXPOSURE TO LIGHT	RATE
		hrs	*millimoles/hr* *		*hrs*	*millimoles/hr* *
1955-C	Unstressed **	12	−3.2	Stressed in darkness ** 36 hrs	11	+3.6
	"	18	−3.7	" " " 60 "	11	+2.8
	.....	..	...	" " " 84 "	11	+2.3
1953-B	Stressed in light 27 hrs	11	−5.5	Unstressed	14	+5.6
	" " " 50 "	12	−3.5	.....	..	...
	" " " 74 "	14	−2.5	.....	..	...
1953-A	Stressed in light 24 hrs	6	−5.1	Unstressed	10	+8.8
	" " " 24 "	16	−5.3	"	18	+6.4
	.....	..	...	Stressed in darkness 24 hrs	7	+4.9
	.....	..	...	" " " 24 "	16	+3.0
1952-A	Stressed in light 24 hrs	8	−4.1	Unstressed	8	+13.3
	" " " 24 "	12	−5.4	"	12	+12.2
	" " " 24 "	17	−4.1	"	18	+8.6
1951-C	Unstressed	16	−5.8	.....	..	...
1951-B	.....	..	...	Unstressed	10	+7.2
	.....	..	...	"	15	+6.5
	.....	..	...	"	20	+5.2

* Data expressed in terms of 1 kilo of initial fresh wt.

** "Unstressed" leaves had been cultured in darkness or in light for the indicated period after being picked. "Stressed" leaves were cultured in light or in darkness for the indicated period and were then transferred to the other situation for the periods indicated under "Total exposure." The change in composition took place during this subsequent exposure at the rate shown.

TABLE III

RATE OF CHANGE OF MALIC ACID CONTENT OF LEAVES OF BRYOPHYLLUM CALYCINUM DURING EXPOSURE TO DARKNESS OR TO LIGHT

SAMPLES	CONDITION BEFORE TREATMENT	TOTAL EXPOSURE TO DARKNESS	RATE	CONDITION BEFORE TREATMENT	TOTAL EXPOSURE TO LIGHT	RATE
		hrs	*milli-moles/hr* *		*hrs*	*milli-moles/hr* *
1955-C	Unstressed **	12	+5.0	Stressed in darkness ** 36 hrs	11	- 3.6
	"	18	+4.0	" " " 60 "	11	- 2.2
	.....	..	...	" " " 84 "	11	- 1.6
1953-B	Stressed in light 27 hrs	11	+2.1	Unstressed	14	- 6.5
	" " " 50 "	12	+1.6	.....	..	...
	" " " 74 "	14	+1.1	.....	..	...
1952-A	Stressed in light 24 hrs	8	+8.1	Unstressed	8	-11.0
	" " " 24 "	12	+7.2	"	12	- 9.9
	.....	..	...	"	18	- 7.1
1951-C	Unstressed	16	+3.2	.....	..	...
1951-B	.....	..	...	Unstressed	10	- 8.7
	.....	..	...	"	15	- 7.5
	.....	..	...	"	20	- 5.5

* Data expressed in terms of 1 kilo of initial fresh wt.
** See footnote to table II.

Notwithstanding the continuous loss of malic acid which began after 18 hours in darkness, the samples when later transferred to light lost malic acid at a greatly accelerated rate so that, after 11 hours in light, the quantity had decreased considerably below that present when the leaves were picked. In these samples, indeed, malic acid had become almost a minor acid component.

The leaves exposed to continuous light lost malic acid rapidly for 14 hours and slowly thereafter although in no case did the level to which it diminished drop as low as that seen in the transferred leaves of the parallel experiment. The irregular fluctuations which occurred after 24 hours are almost certainly greater than the experimental error, and may reflect minor irregularities in the conditions (e.g., temperature) in the culture room. The outstanding result is, however, that after transfer to darkness, the enzyme systems of these leaves were unable to synthesize malic acid in sufficient amounts to restore even one-half of the quantity present when the leaves were picked. Different lots of Bryophyllum leaves may be expected to contain between 200 and 300 meq of malic acid per kilo of fresh weight when collected at daybreak, so that the present samples, which contained 247 meq per kilo, were of about average composition. Inasmuch as the malic acid content of leaves that have been excised at daybreak and exposed to the normal diurnal variation in illumination is restored nearly to its initial level during the subsequent night period (8), it is clear that exposure to an artificially prolonged period of illumination adversely affects the mechanisms concerned in the synthesis of malic acid during a subsequent period of darkness.

In table III are collected data on the rates at which malic acid is synthesized in darkness or at which it disappears in light in a number of sets of samples of Bryophyllum leaves. In normal leaves picked at nightfall (i.e., unstressed and held in darkness), the rates of synthesis range from 3 to 5 millimoles per hour per kilo, and the unusually vigorous set marked 1952-A, which had contained 299 meq of malic acid at daybreak, increased in malic acid content at the rate of 8 millimoles per hour even after the leaves had been exposed to light for 24 hours. Nevertheless, stress brought about by long exposure to light (samples 1953-B) diminishes the rate at which malic acid is synthesized after transfer of leaves to darkness, and the effect is more pronounced the longer the period of stress.

On the other hand, leaves picked at daybreak (unstressed and exposed to light) lose malic acid at the rate of from about 6 to 11 millimoles per hour. The effect of the stress occasioned by long exposure to darkness is clearly evident in the much diminished rates at which malic acid disappeared in a subsequent light period, as is shown by the successively lower rates of loss of malic acid in the stressed leaves of samples 1955-C. However, inasmuch as the level of malic acid in these samples had diminished materially during prolonged culture in darkness (fig 2), the lower rates of loss after transfer to light do not necessarily imply impairment of the metabolic system concerned.

The response of citric acid to change of the conditions of illumination of Bryophyllum leaves as a rule is less spectacular than is that of malic acid. In general, however, the responses are parallel and, although they may be of smaller absolute magnitude in terms of the number of milliequivalents of citric acid formed or decomposed, the changes are often substantial in terms of relative amounts. The curve for citric acid at the left of figure 2 illustrates this point un-

usually well. The citric acid in the leaves cultured in darkness increased 6-fold from 13 meq per kilo in the leaves picked in the afternoon to 76 meq after 18 hours in darkness. Malic acid increased nearly 7-fold in the same time. When the leaves were transferred to light after 36 hours in darkness, citric acid dropped from 87 meq per kilo to 25 meq in 11 hours, or to less than one-third of the largest quantity present. The changes that occurred are closely correlated with the changes in starch. The coefficient of correlation between the data for citric acid and for starch in this set of samples was -0.956 ($r = 0.872$ for $P = 0.001$ with 8 degrees of freedom) which is highly significant. The close correlation between the data for malic acid and for starch, which is so striking in experiments on normal diurnal variation (8), is masked in the present instance because of the slow and continuous loss of malic acid which supervened after the leaves had been in darkness for 18 hours.

The leaves exposed to continuous light, the data for which are plotted at the right of figure 2, show the converse of these last relationships. The data for malic acid and for starch are closely related, the coefficient of correlation being -0.921 which is highly significant at the 0.1 % level, whereas the data for citric acid and starch, on the other hand, have a coefficient of correlation of only -0.499 which is not significant even at the 10 % level ($r = 0.549$ for $P = 0.1$ with 8 degrees of freedom). However, the relationship of the behavior of citric acid to that of starch in this instance is confused by an uncertainty regarding experimental conditions to be discussed in a subsequent paragraph.

In previous papers of this series on the metabolism of the organic acids in Bryophyllum leaves, the behavior of citric acid has received only passing attention since the chief concern has been with the malic acid and starch. Sufficient data are now at hand to warrant discussion of the somewhat discordant results that have been obtained. In table IV are data from 7 separate experiments in which the behavior of citric acid has been examined in samples collected by the statistical method and subjected to culture in water under various conditions.

When leaves are sampled in the late afternoon and cultured in water in darkness, citric acid increases for many hours at a remarkably constant rate which averages about 3.8 ± 0.7 millimoles per hour per kilo of initial fresh weight. Even if collected at sunrise and subsequently held in darkness (samples 1949-C), accumulation of citric acid at about this same rate continues for at least 7 hours. The data for samples 1949-B represent two successive cycles of 9 hours of darkness, each of which was preceded by a full day of exposure of detached leaves to sunlight; the conditions are reckoned to be "unstressed" since the exposure to light was normal in length. If, however, the leaves are subjected to stress by prolonged exposure to light (samples 1953-B), the rate at which citric acid is formed is somewhat diminished although it is still substantial.

The behavior of citric acid in leaves picked at day-

TABLE IV

RATE OF CHANGE OF CITRIC ACID CONTENT OF LEAVES OF BRYOPHYLLUM CALYCINUM DURING EXPOSURE TO DARKNESS OR TO LIGHT

SAMPLES	CONDITION BEFORE TREATMENT	TOTAL EXPOSURE TO DARKNESS	RATE	CONDITION BEFORE TREATMENT	TOTAL EXPOSURE TO LIGHT	RATE
		hrs	*millimoles/hr* *		*hrs*	*millimoles/hr* *
1955-C	Unstressed **	12	+4.0	Stressed in darkness ** 36 hrs	11	−5.7
	"	18	+3.5	" " " 60 "	11	−5.4
	.....	..	...	" " " 84 "	11	−4.1
1953-B	Stressed in light 27 hrs	11	+2.2	Unstressed	14 †	+0.09
	" " " 50 "	12	+3.3	"	27 †	+0.07
	" " " 74 "	14	+2.8	.....	..	...
1952-A	Stressed in light 24 hrs	8	+3.3	Unstressed	8 †	−1.3
	" " " 24 "	12	+3.0	"	12 †	−1.7
1951-C	Unstressed	16	+3.8	.....	..	...
	"	24	+3.1	.....	..	...
1951-B	.....	..	...	Unstressed	10 †	−0.5
	.....	..	...	"	15 †	−0.4
1948-B	Unstressed	9	+4.5	Unstressed	10	−3.6
	"	9	+4.7	"	15	−3.1
1949-C	Unstressed	7 ††	+3.8	.....	..	...
	"	14 ††	+2.7	.....	..	...

* Data expressed in terms of 1 kilo of initial fresh wt.
** See footnote to table II.
† In artificial light.
†† Leaves collected at sunrise and cultured in darkness.

break and exposed to light is, however, by no means always predictable. The stressed leaves of samples 1955-C and the unstressed leaves of samples 1949-B (table IV), which were exposed in the greenhouse, lost citric acid at rates comparable with those at which it is synthesized in darkness. The leaves of samples 1953-B, 1952-A and 1951-B which were exposed to artificial light at controlled temperature and humidity immediately after being picked at daybreak lost citric acid much more slowly; indeed, those of samples 1953-B apparently gained slightly (fig 2). A question is at once raised whether the situation under the conditions of artificial illumination was truly comparable with that which obtained in the greenhouse where samples 1955-C and 1949-B were treated. The conditions with respect to temperature were certainly different. The leaves under the artificial lights were maintained near 22° C as observed on thermometers placed close to the surfaces of the leaves, although this was at best only an approximate indication of the temperature within the tissues. The temperature of the greenhouse, on the other hand, varied widely and, since the experiments were done on warm sunny days, rose to the vicinity of 38° C or even higher in the early afternoon. The mean temperature over the whole experimental period was doubtless considerably higher than that in the temperature-controlled room.

That either the intensity or the quality of the artificial light was responsible for the difference in behavior seems less probable. The leaves of samples 1953-B increased rapidly and extensively in both organic solids and starch as is shown in figure 1, and the same is true of samples 1951-B (3). Under the same conditions of illumination, the starch content of samples 1953-A (table II) showed the most extensive and rapid increase that has been observed in this laboratory although the increase of organic solids was not striking (1). Accordingly, the light intensity in the room used was adequate for photosynthetic reactions and for the reactions concerned in the formation of starch, and there is no indication that there was an insufficiency with respect to quality. It is obvious that the discrepancies can only be cleared up by suitably planned additional experiments.

The behavior of isocitric acid in the present samples (fig 2) is of special interest. It has been pointed out previously that isocitric acid remains constant within the limits of the error of measurement in Bryophyllum leaves exposed to the normal diurnal variation of illumination (8), although a possibly significant loss occurs on long exposure to light (3). The curves for the behavior of isocitric acid in figure 2 show that, during culture in darkness, this substance followed a somewhat irregular course. However, the mean content of the 7 samples was 173 ± 5.7 meq per kilo. The variation was thus only 3.3 % of the quantity measured, and accordingly, there is no convincing evidence of a change in isocitric acid during the treatment of the leaves which was significantly greater than the variability of the observations. On transfer to light, however, there was a significant decrease in the amount of isocitric acid in these samples. The 3 observations are consistent and are supported by the data of the curve at the right of the figure which show the behavior of isocitric acid in the leaves cultured in continuous light. In these samples, isocitric acid diminished continuously although slowly. A regression line calculated for the 7 samples cultured in light shows that approximately 9 % of the isocitric acid present at the start disappeared during the 74 hours of the culture period. This is greater than the analytical error, and the conclusion may be drawn that isocitric acid is to some extent utilized under these conditions. The tentative conclusion in an earlier paper (3) is thus confirmed. However, in these leaves, transfer to darkness brought about only negligibly small changes which were well within the error of the observations.

Discussion

Effects of Stress: The effect of prolonged exposure to light or to darkness upon the rate at which starch disappears or is synthesized after transfer of the leaves from one situation to the other is shown by the data for samples 1955-C and 1953-B in table II. These rates furnish measures of the slopes of the broken lines in figure 1. The progressive change in the rates represents the effect of the increase in the stress to which the physiological systems had been exposed. The analogous data for the rates at which malic acid underwent change are given for the same sets of samples in table III, and those for citric acid in table IV. These rates are indicated by the slopes of the broken lines in figure 2. In all instances, with the exception of the data for the rate of change of citric acid in the leaves that had been stressed in light and subsequently transferred to darkness, the rates diminished with the increase in the period of culture before the transfer was made. The decrease in the rates of the reactions furnishes evidence of progressive impairment of the ability of the enzymatic systems involved to recover from the effects of the stress.

The close inverse correlation that has repeatedly been observed between the data for decrease of malic acid and increase of starch in Bryophyllum leaves exposed to light, as well as for the apparent reversal of this relationship in leaves exposed to darkness, has been advanced as evidence in favor of the view that a sequence of chemical reactions occurs whereby actual transformation of the one substance into the other takes place. The present data reflect disturbances in the efficiency or speed of these reactions brought about by the onset of what has been designated "stress." In general, the speed of chemical reactions is influenced by temperature, and by the relative proportions of the reactants involved. With enzymatically catalyzed reactions, it is also a function of the affinity of the substrate for the enzyme, a quantity which is measured by the Michaelis constant. The speed of reactions is likewise affected by the presence of inhibitors and, in addition, if the concentration of one or more of the essential enzymes or co-

factors is changed, a corresponding change in reaction speed would occur. In the present experiments, there was no reason to suppose that the temperature, at which the three samples in each set subjected to transfer were treated, diminished in a systematic way so as to retard progessively the rates at which the chemical reactions proceeded. On the contrary, the physical conditions after each transfer were duplicated as closely as possible. With respect to the possibility that the speed of the reactions diminished because of progressive failure of the supply of one of the reactants, it may be pointed out that malic acid did indeed slowly drop to a low level during prolonged culture of the leaves in darkness (fig 2), and this may have affected the rate at which starch was synthesized in these samples after transfer to light. On the other hand, in the samples cultured in light, malic acid was at approximately the same low level in all three samples which were transferred to darkness, and starch was at a high level in all, yet a progressive change in the rates of reaction occurred. Accordingly, it is difficult to account for the decrease in reaction rate after transfer in terms of a deficiency of one of the reactants.

That change occurred in the Michaelis constants of individual enzymes as the period of culture increased is highly improbable; the affinity of an enzyme for its substrate is a property characteristic of the protein itself and, within the limits of present knowledge, is fixed in magnitude. One is left, therefore, with two hypotheses to account for the observed progressive change in the rates of the reactions; the possibility of the generation of inhibitory substances, or the possibility of the progressive destruction of one or more of the enzymes or co-factors essential for the catalysis of the reactions. Whether or not inhibitory substances are gradually formed must be left for future investigation, and at present little information regarding co-factors is available. In any case, however, the most likely hypothesis is that a slow destruction of the enzymes occurred. Information that may bear on this point was obtained by an examination of the protein nitrogen. It has been mentioned that a plot of the data suggested that a progressive loss of protein took place throughout the period of culture. The regression line calculated for the 7 points which represent the protein nitrogen of the leaves cultured in darkness indicated that about 6.5 % of the protein had disappeared at the 84-hour point when the third sample was transferred to light. The regression line for the protein nitrogen of the samples cultured in light suggested that about 3.8 % of the protein had disappeared at the 74-hour point when the third transfer to darkness was made. That these losses of protein reflect the gradual decomposition of enzymes essential for the chemical reactions which occurred is indeed speculative, but it can be concluded that such an event is in conformity with the observations on the changes in protein content.

Quantitative Relationships between Starch and Acids: The hypothesis that starch is converted into organic acids in darkness and acids are converted into starch in light can be tested by comparison of the quantities of the presumed reactants which underwent change subsequent to transfer from the one to the other condition of illumination. The essential information is collected in table V. In order to avoid all considerations of chemical mechanisms, the data are expressed in terms of millimoles of carbon, and the figures are arranged so as to answer the question: Was sufficient carbon supplied, by the component that diminished in quantity, to account for the increase in the component to which it was presumably converted? The upper part of table V refers to the leaves which were cultured in darkness and transferred to light, the lower to the leaves subjected to the converse treatment. The data for the changes in starch are given in gm per kilo in column 3 in order to furnish an easily appreciated measure of the direction and magnitude of the phenomena. Column 7 gives the ratio between the sum of the carbon involved in the change in the quantity of malic and citric acids to the carbon involved in the change in the amount of starch. For the unstressed leaves cultured in darkness, the ratio of 1.46 indicates that appreciably more of these acids were formed than could have arisen from the starch. Carbon from some other source must have entered the system, and such observations as those of Thurlow and Bonner (9) and of Varner and Burrell (10) with radioactive carbon dioxide indicate that fixation of carbon can account for a part at least of the extra carbon found in these acids. The rather precise agreement between the loss of carbon from starch after 12 hours and the corresponding gain of carbon of malic acid is, however, worthy of attention.

At the end of 18 hours, although the rate of loss of starch had been maintained, the rate of gain of the acids had diminished, with the result that the ratio of the quantities of carbon approached unity. Thus the quantities of carbon involved in the overall reactions which had occurred during 18 hours are in conformity with the hypothesis.

After transfer of the leaves to light, starch was synthesized and malic and citric acids diminished. The ratios between the quantities of carbon involved in these reactions in each of the 3 samples remained essentially constant notwithstanding the wide differences in the amounts of the substances concerned, and thus also conform with the hypothesis of actual transformation. That the ratios are slightly greater than unity suggests that the transformation was somewhat short of being quantitative, that is to say, a little of the malic or citric acid or possibly of both was converted into products other than starch.

The data in the lower part of table V show that considerably more carbon appeared in the starch formed during the first 14 hours of culture in light than can be accounted for by the carbon of the acids that disappeared. If a correction is applied to the amount of starch synthesized by subtracting the weight of the organic solids that were formed

(see data in parentheses), these solids being assumed to represent photosynthesized starch, the ratio is somewhat increased and, when this calculation is carried out for the leaves that had been cultured for 27 hours in continuous light, reaches 1.07. A previous observation (3) is thus confirmed. It may be concluded, therefore, that unstressed leaves cultured in light behave in a manner which conforms with the hypothesis that much of the starch formed may arise from chemical transformation of the organic acids.

The leaves that had been stressed by prolonged exposure to light before transfer to darkness were, however, no longer able to make efficient use of the starch for the synthesis of acids. The ratio between the quantities of carbon is much depressed. Attention has already been drawn to the point that the increase in malic acid was far less striking (fig 2 and table III) than that in unstressed leaves both in amount and in the rate at which the reaction occurred. The damage appears to have been especially severe to the systems involved in the synthesis of this substance, although the systems concerned with the formation of citric acid do not seem to have been so gravely affected. No information was obtained regarding the fate of that part of the starch which did not appear to contribute to the acid metabolism.

The present investigation has provided further evidence on the interrelationships of starch and organic acids in Bryophyllum leaves in light and darkness inasmuch as it is shown that almost quantitative transformation of the one into the other is within the bounds of possibility. The crux of the problem is, however, the elucidation of the mechanisms whereby these chemical events are brought about. Upon this matter, there is nothing to offer at the present time save speculation. It seems certain, however, that a careful study of the enzyme systems of this species will ultimately lead to at least a partial understanding of the extraordinary chemical behavior of crassulacean plants.

Summary

Initially identical samples of excised leaves of *Bryophyllum calycinum* have been subjected to prolonged culture in water in darkness, individual samples being transferred to light at intervals. A second set of samples was subjected to the converse conditions, the object being to test the capacity of the leaves to resume the normal rhythm of variation in composition with respect to starch and organic acids.

The effect of prolonged culture either in darkness or in light was to set up a condition which is interpreted as representing a steadily increasing degree of physiological stress. This condition was characterized by the diminishing speed at which the major chemical changes in composition subsequent to transfer were

Table V

Molar Relationship between the Carbon of Starch and of Malic and Citric Acids Involved in Changes in Composition Which Occur in Bryophyllum Leaves Subjected to Culture in Water

Condition before treatment	Hrs of preliminary culture	Δ Starch, gm *	A Δ Starch carbon, millimoles *	B Δ Malic acid carbon, millimoles *	C Δ Citric acid carbon, millimoles *	Ratio (B+C)/A
Unstressed: cultured in darkness (Samples 1955-C)	12	− 6.3	−234	+240	+ 98	1.46
	18	−10.8	−401	+290	+127	1.04
Stressed by culture in darkness for period shown: Change after transfer to light for 11 hrs	36	+ 6.5	+239	−160	−125	1.19
	60	+ 4.9	+182	− 95	−119	1.17
	84	+ 4.0	+149	− 68	− 90	1.06
Unstressed: cultured in light (Samples 1953-B)	14	+12.7	+469 (+440) **	−363	+ 2.6	0.77 (0.82) **
	27	+15.9	+590 (+353) **	−385	+ 5.3	0.64 (1.07) **
Stressed by culture in light for period shown: Change after transfer to darkness for 11, 12 and 14 hrs, respectively	27	− 9.8	−362	+ 93	+ 53	0.40
	50	− 6.8	−250	+ 77	+ 79	0.62
	74	− 5.7	−212	+ 59	+ 77	0.64

* Data expressed in terms of 1 kilo of initial fresh wt.
** Corrected for increase of organic solids assumed to represent starch.

observed to take place as the culture period was prolonged. The data refer particularly to the synthesis and decomposition of starch, and the correlated reciprocal changes in malic and citric acids.

During the culture period, the total protein of the leaf slowly diminished in amount. It is tentatively suggested that the changes in the rates at which the leaves are able to recover from the stressed condition are correlated with the loss of protein and specifically with the gradual, although only partial, destruction of the enzymes essential for the chemical transformations to occur. The enzymes concerned with the metabolism of malic acid appear to have been especially sensitive to prolonged culture in light.

An examination of the data for the reciprocal changes in starch and organic acids showed that, in general, approximately the correct quantity of carbon was supplied by the component which diminished in amount to account for the carbon of the component which was synthesized. However, this relationship did not hold for the synthesis of organic acids in leaves which had been stressed by prolonged culture in light and were then placed in darkness. In these leaves, there was a marked deficiency in the synthesis of organic acids.

In contrast to its behavior in Bryophyllum leaves exposed to light under greenhouse conditions, citric acid was observed to diminish slowly, if at all, when the leaves were exposed to artificial light at a controlled temperature in the vicinity of 20° C.

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THE DARK FIXATION OF CO_2 BY SUCCULENT LEAVES: THE FIRST PRODUCTS[1,2]

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It has long been realized that green leaves have the ability to fix CO_2 in the dark to form organic acids with the concomitant loss of carbohydrate stores. A group of plants known as the succulents is characterized by a particularly active dark fixation of CO_2. In the case of the succulents, the organic acids synthesized in the dark are transformed to carbohydrates during a subsequent light period (4). Despite the considerable amount of work which has been done on succulent metabolism (11), the exact metabolic pathways by which the dark fixation of CO_2 proceeds have not, hitherto, been elucidated.

The techniques of paper chromatography and radioautography developed by Calvin, Benson, and their co-workers (3), to determine the pathway of $C^{14}O_2$ in photosynthesis are particularly applicable, with modifications, to the study of the dark fixation of $C^{14}O_2$ by succulents. That $C^{14}O_2$ is incorporated into the organic acids of succulents has been demonstrated by Thurlow and Bonner (13) who exposed *Bryophyllum crenatum* leaves to $C^{14}O_2$ for 60 hours. By means of a gross chemical separation of the prod-

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ucts, they found that about 50 % of the activity was associated with the organic acid fraction, the remainder with amino acids, sugars, and insoluble materials. More refined techniques for the separation of organic acids on silica gel columns were employed by Varner and Burrell (16) in their study of $C^{14}O_2$ fixation by excised *B. calycinum* leaves. After two hours of dark fixation, radioactivity was found in malic, oxalic, succinic, citric, and isocitric acids. The malate contained 76 % of the activity fixed in the organic acid fraction, confirming that this compound is the principal acid concerned with acidification in the succulents. Ranson (12) who used paper chromatography, $C^{14}O_2$, and radioautography identified aspartate and glutamate as well as the above-named organic acids in 14-hour experiments with excised Kalanchoë leaves.

In all of the experiments outlined above, relatively long dark CO_2 fixation periods were used and mixtures of products were obtained. It should be possible to identify the product or products of the initial carboxylation reaction by shortening the reaction period. This paper will describe experiments in which we have been able to identify the products of the 6-second dark fixation of $C^{14}O_2$ by excised *B. calycinum* leaves as malate and aspartate. It is suggested that the reaction mechanism for the process involves oxaloacetate as the common, but transient precursor. Evidence for the occurrence of oxaloacetate as well as the presence of the enzyme mediating the synthesis of this compound from PEP[3] and CO_2 in the leaves of *B. calycinum* supports the postulated mechanism.

Materials and Methods

CO_2 Dark Fixation Apparatus: An apparatus was devised in which succulent leaves were exposed to $C^{14}O_2$ in complete darkness, and which permits the leaves to be homogenized in situ after very short exposure intervals. The apparatus is shown in figure 1. A stainless steel micro-Waring blendor was fitted with a large two-holed rubber stopper. The blades were modified, as indicated, to insure total disruption of the leaves. All glass connections were coated with an opaque wax to exclude light. Approximately 1 gm of intact excised leaves are placed around the blade, the stopper securely clamped in place, a vacuum created in the chamber by means of an aspirator, and $C^{14}O_2$, previously generated from $BaCO_3$ and collected in the trap, drawn into the chamber. To terminate the reaction, a vacuum is again created in the chamber and 30 ml of boiling 80 % ethanol drawn into the chamber with simultaneous homogenization. The homogenate was filtered, extracted with Skelly A solvent to remove chlorophyll and carotenoids, and concentrated to a final volume of 2.0 ml under reduced pressure.

[3] The following abbreviations are employed: PEP, phosphoenolpyruvate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; DPN$^+$, oxidized diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; TRIS, tris (hydroxymethyl) aminomethane; OA, oxaloacetate; ITP, inosine triphosphate; IDP, inosine diphosphate.

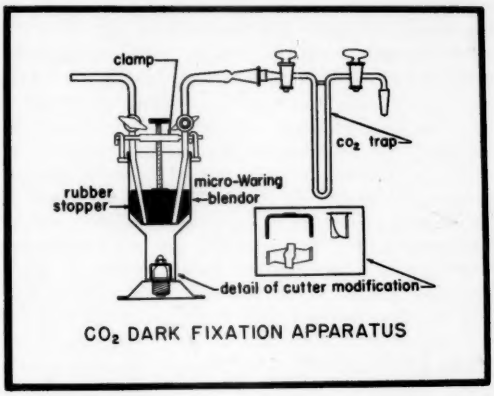

Fig. 1. Apparatus designed for the exposure of intact leaves to $C^{14}O_2$ in complete darkness.

B. calycinum from the clone maintained by the Division of Biology, California Institute of Technology, were obtained through the generosity of Dr. James Bonner and Mr. Charles Newman. The plants were 10 to 15 cm tall when used. Only the young leaves, 1 to 2 cm in length, near the apex of the stem were used. No special care was exercised in maintaining environmental conditions of light or temperature during growth. Prior to use, the whole plants were exposed to sunlight for at least four hours to insure that they possessed an appreciable carbohydrate store.

Chromatographic and Radioautographic Methods: The procedures of Benson et al (3) were used with slight modification. Aliquots (0.1 to 0.2 ml) of the concentrated ethanolic extract were chromatographed on Whatman No. 1 (18″ × 22″) by descending chromatography. The first solvent was 80 % phenol : 20 % water (w/w), the second solvent *n*-butanol (79) : acetic acid (19) : water (50) (v/v). Compounds were located by radioautography made with "no-screen" x-ray film. Activity of each area was determined using an end window Geiger tube directly on the paper. Authentic samples of suspected compounds were cochromatographed with the extract and identification made by superposition. Amino acids were located with the ninydrin in collidine spray of Levy and Chung (8); organic acids with a mixed indicator spray of 3 gm brom-phenol blue–1 gm methyl red per 1000 ml of 95 % ethanol.

Preparation of Enzyme: Homogenates from leaf tissue were prepared by the method of Waygood (18). The leaves are infiltrated with 1 % NH_4OH to neutralize the acidic compounds present in the leaf which will otherwise inactivate the enzyme during grinding. Ten gm of infiltrated leaves were ground with sand in a cold mortar with 10 ml of 0.1 M TRIS buffer, pH 7.5. The homogenate was centrifuged at 500 × g for 5 minutes to remove cell debris and the cloudy

supernatant assayed for PEP-carboxylase activity by the method of Bandurski and Greiner (2).

ISOLATION OF OXALOACETATE: Since oxaloacetate is present in the leaf tissue in very low concentrations and is very labile and readily destroyed when the leaf tissue is homogenized, a satisfactory procedure for trapping the oxaloacetate with carrier and 2,4-dinitrophenylhydrazine was carried out as follows: leaves were permitted to fix $C^{14}O_2$ in the dark for 20 minutes. Fifty ml of saturated 2,4-dinitrophenylhydrazine in 2 *N* HCl in which 100 mg oxaloacetate were dissolved immediately before use were drawn into the reaction vessel and the tissue homogenized. The reaction mixture was immediately filtered, and the supernatant set aside at room temperature to permit crystallization of the phenylhydrazone derivative. The phenylhydrazone was isolated and recrystallized to constant specific activity following the procedures outlined by Bandurski and Greiner (2).

RESULTS AND DISCUSSION

Radioautography of a chromatogram of a leaf extract after a fixation period of 6 seconds is shown in figure 2. Only two products have incorporated $C^{14}O_2$ in this time. These are malate and aspartate. The radioactivity in each of these two compounds was determined for successively longer periods of exposure to $C^{14}O_2$. These activities expressed as percent of total activity in the extract for each exposure time are plotted in figure 3. Extrapolation of these values to zero time shows, however, that there might be some common precursor to the malate and aspartate that is too labile to appear on the chromatograms. A possible hypothesis is that oxaloacetate may be such a precursor. It is also possible that aspartate and malate would incorporate label via independent pathways.

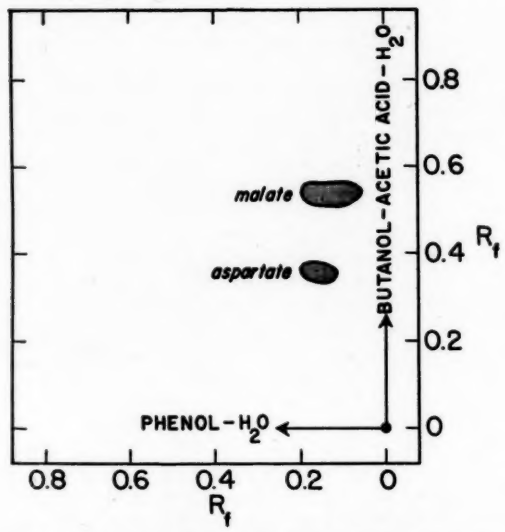

FIG. 2. Tracing of radioautogram obtained from extract of *Bryophyllum calycinum* leaves after 6-sec exposure to $C^{14}O_2$.

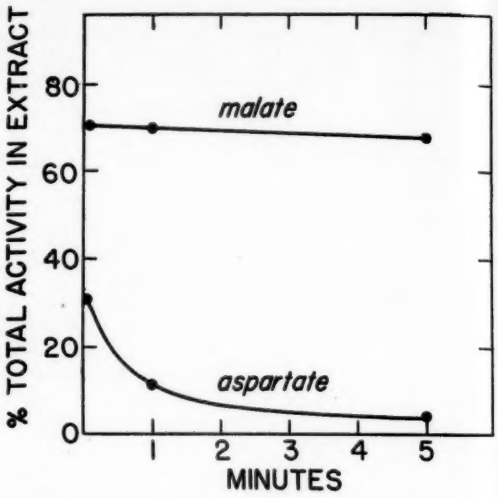

FIG. 3. Radioactivity of malate and aspartate expressed as percent total activity in extract as a function of time.

Much speculation has appeared in the literature concerning the mechanism for the initial fixation of CO_2 in the succulents. Several workers have proposed that the responsible system is the "malic enzyme" (9).

(*1*) $CO_2 + \text{pyruvate} + \text{TPNH} + H^+ \rightarrow \text{malate} + \text{TPN}^+$

Others have favored the Wood-Werkman reaction (19).

(*2*) $CO_2 + \text{pyruvate} \rightarrow \text{oxaloacetate}$

It should be pointed out that this reaction is a purely speculative one and has not, as yet, been experimentally verified. Utter and Wood (15) demonstrated that in the presence of ATP and Mg^{++}, pyruvate could be carboxylated to yield oxaloacetate

(*3*) $\text{pyruvate} + CO_2 \xrightarrow[Mg^{++}]{ATP} \text{oxaloacetate}$

The experiments of Bandurski and Greiner (2) with spinach leaves and of Utter and Kurahashi (14) with liver have clarified the mechanism for the carboxylation of PEP. The enzyme phosphoenolpyruvylcarboxylase from spinach leaves catalyzes the reaction

(*4*) $\text{PEP} + CO_2 \rightarrow \text{oxaloacetate} + \text{orthophosphate}$

It is an essentially irreversible reaction and is not dependent upon any nucleotide coenzyme (1). The liver system, on the other hand, is readily reversible and requires the participation of nucleotide coenzymes

(*5*) $\text{PEP} + CO_2 + \text{IDP} \leftrightarrows \text{ITP} + \text{oxaloacetate}$

Thus, the direct phosphorylation of pyruvate

(*6*) $\text{pyruvate} + \text{ATP} \rightarrow \text{PEP} + \text{ADP}$

TABLE I

ACTIVITY OF PEP-CARBOXYLASE IN BRYOPHYLLUM CALYCINUM EXTRACT

CONDITION	CPM FIXED IN REACTION MIXTURE
Enzyme + PEP	5,430
Enzyme − PEP	30
Boiled enzyme + PEP	0

Each tube contained 60 micromoles TRIS hydrochloride, pH 7.5, 20 micromoles $MgSO_4$, 100,000 cpm $NaHC^{14}O_3$, 0.2 ml enzyme, 6 micromoles PEP. Total volume 1.5 ml. Incubated 60 min at 37° C. Reaction stopped with 0.1 ml 1 *N* HCl and unreacted $C^{14}O_2$ removed by bubbling N_2 through the mixture. An 0.2-ml aliquot of the reaction mixture was counted with an end-window Geiger tube.

linked to either reaction *4* or reaction *5* constitutes a likely reaction mechanism for reaction *3*.

Both the reversible and irreversible carboxylation reactions have been found together in wheat germ (10). That the enzyme of Bandurski's experiment is present in succulent tissue is shown by the data of table I which concerns results from a typical experiment with a homogenate, prepared as indicated above. An estimate of the in vivo activity of PEP carboxylase in *B. calycinum* leaves is impossible because of the losses in activity incurred during preparation.

The presence of oxaloacetate in plant tissues in rather low concentrations has been noted by Virtanen (17). Virtanen also made the observation that the level of oxaloacetate rises in the light and falls in the dark. Bradbeer (5) has obtained evidence for the presence of oxaloacetate in Kalanchoë leaves. He was further able to show that oxaloacetate incorporates $C^{14}O_2$ during a 6-hour dark fixation. We have confirmed Bradbeer's results by permitting leaves of *B. calycinum* to fix $C^{14}O_2$ and by subsequently killing the tissue with carrier oxaloacetate and isolating the 2,4-dinitrophenylhydrazone as described above. Table II presents data from such an experiment. Characterization of the 2,4-dinitrophenylhydrazone of oxaloacetate was made by elemental analysis and cochromatography with an authentic sample confirmed its identity.

Thus it is clear, from the evidence of figure 2, that a single compound gives rise to both malate and aspartate, and the identification of both oxaloacetate and the enzyme, PEP carboxylase, which mediates the synthesis of oxaloacetate, suggests the following scheme for the initial reactions of dark CO_2 fixation by succulent leaves:

TABLE II

CRYSTALLIZATION OF THE 2,4-DINITROPHENYLHYDRAZONE OF OXALOACETATE TO CONSTANT SPECIFIC ACTIVITY

NO. OF CRYSTALLIZATIONS	WT (MG)	CPM/MG
1	95.0	30.1
2	67.1	27.2
3	40.6	27.0

Conditions of the experiment are described in the text. The phenylhydrazone was recrystallized from ethyl acetate by the addition of hexane. Radioactivity was determined on an aliquot with an end-window Geiger tube.

(7)

$$\text{Carbohydrate} \xrightleftharpoons{Glyc} \text{PEP}$$

$$\text{PEP} \xrightarrow[PC]{+CO_2} \text{oxaloacetate}$$

$$\text{Oxaloacetate} \begin{cases} \xrightleftharpoons[MD]{} \text{malate} \\ \xrightarrow[T]{+NH_3} \text{aspartate} \end{cases}$$

Glyc = glycolysis *MD* = malic dehydrogenase
PC = PEP carboxylase *T* = transaminase

Such a mechanism has been proposed by Tchen and Vennesland (10). The alternative scheme involving malic enzyme in the initial reaction appears unlikely despite the fact that net dark fixation of small amounts of malate is possible (7) by this system. If malate were the first product, it would be difficult to conceive of its oxidation to oxaloacetate and subsequent transamination to aspartate. The equilibrium for the reaction

(8) $$\text{malate} + DPN^+ \rightarrow OA + DPNH + H^+$$

greatly favors the reduction of OA (6). The transaminase reaction is essentially a freely reversible chemical equilibrium. Since succulents have the ability to accomplish a great deal of net CO_2 fixation, it seems more probable that the reaction scheme (7) is operative.

SUMMARY

1. The major products of the dark fixation of $C^{14}O_2$ by *Bryophyllum calycinum*, a typical succulent, have been identified as malate and aspartate during a 6-second fixation period.

2. The kinetics of the labeling from these two compounds during longer periods of dark fixation indicate that they must arise from a common precursor. It is suggested that this precursor may be oxaloacetate.

3. Radioactive oxaloacetate has been identified in leaves exposed to $C^{14}O_2$. The enzyme catalyzing the carboxylation of PEP to oxaloacetate has been identified in *B. calycinum* homogenates.

4. A scheme to account for the initial fixation of CO_2 during the dark acid metabolism of succulents is proposed.

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PERMEABILITY OF VICIA FABA ROOT SEGMENTS TO WATER AS MEASURED BY DIFFUSION OF DEUTERIUM HYDROXIDE[1,2]

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In experiments with Avena coleoptiles reported previously (7) the time required for internal concentrations of deuterium hydroxide (DHO) to attain half of the external concentrations was found to be 8 or 9 minutes. It was found in those experiments that segments of coleoptiles killed by treatment with 2,4-dinitrophenol (DNP) exhibited only a slight increase in permeability to water, the half time being decreased to about 6.5 minutes in dead coleoptile segments. This contrasts with results obtained by Brouwer (1) and Kramer (4) with roots, and results of Wartiovaara (12) with Tolypellopsis, all of these workers finding a large increase in permeability to water after killing. These workers concluded that most of the resistance to water movement in roots resides in the cytoplasm but the results of the experiments with Avena coleoptiles suggest that in the latter most of the resistance to water movement resides in the cell walls. A series of experiments similar to those on Avena coleoptiles was, therefore, performed on roots in order to learn if roots behave differently from coleoptiles. It will be shown below that the half time of diffusion of water in *Vicia Faba* root segments is less than one-tenth that in Avena coleoptile segments and that killing either by DNP treatment or by heat treatment results in a considerable decrease in the half time, i.e., permeability increases.

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Materials and Methods

The material used in this work consisted of 10-mm segments cut 3 to 4 mm above the apices of secondary roots of *Vicia Faba*. The diameter of the segments was about 0.5 mm. The *Vicia Faba* (var. Windsor Long Pod) seedlings were grown as follows. About 120 seeds were soaked overnight in an enameled tray containing a shallow layer of distilled water. The seed coats were removed and seeds which appeared healthy, i.e., not attacked by parasites, were planted on water-soaked and drained vermiculite and covered by wet vermiculite in a stainless steel tray. Four

days later seedlings were removed from the vermiculite and were rinsed and transplanted to glass cylinders containing ½ strength Hoagland solution. Solutions were aerated by compressed air passing through sinter glass aerators. Four days after transplanting, the tips of the primary roots were cut off. Six days later water was added to the cylinders. Seventeen days after soaking the seeds, the roots were harvested for experimental use. Each part of each experiment was performed using about 50 segments in 15 ml of solution.

The experiments were carried out with 0.5 % deuterium oxide (D_2O) prepared from 99.5 % D_2O obtained from Stuart Oxygen Company by authorization of the U. S. Atomic Energy Commission. Groups of root sections were placed in DHO solutions for various periods of time. Solutions were made up in 0.005 M KH_2PO_4 at pH 5.0 to 5.3. After the desired time interval the group of segments was poured on a piece of Whatman No. 1 filter paper which had been placed on a porcelain filter funnel and the surfaces of the segments were dried externally by applying suction and by placing another piece of filter paper on top of them. When dry, the sections were quickly transferred to a ground glass stoppered vial which was used as the distilling vessel for removal of the tissue water by lyophilization as described earlier (7). The DHO content of the tissue water was determined by mass spectrometry as described by Washburn et al (13). The precision of measurement is greater than ± 0.01 atom percent over the range of DHO concentrations used in this work.

The data are presented as mole percent DHO in the tissue water as a function of time of exposure to the isotopically labeled solutions.

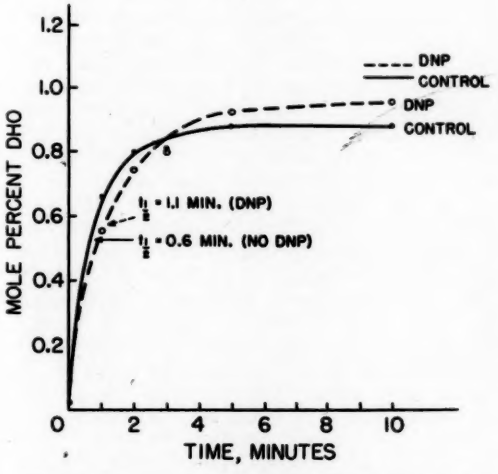

FIG. 1. Effect of 5×10^{-4} M 2,4-DNP pretreatment on inward diffusion of DHO into *Vicia Faba* root sections. External solution concentrations indicated by horizontal lines. Half-times indicated by arrows. 0.005 M KH_2PO_4 pH 5 throughout.

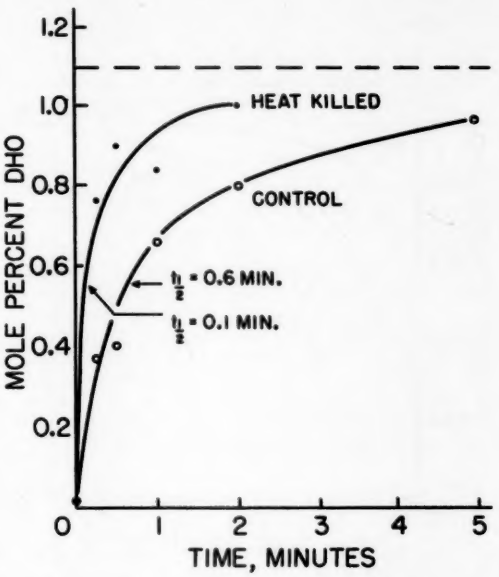

FIG. 2. Effect of killing by heat on subsequent inward diffusion of DHO into *Vicia Faba* root sections. External solution concentration indicated by horizontal line. Half-times indicated by arrows. 0.005 M KH_2PO_4 pH 5 throughout.

RESULTS

The data of figure 1 show that the permeability to water of *Vicia Faba* roots is much higher than that of Avena coleoptiles, the half time of diffusion of DHO into the roots being only 0.6 minute compared to 8 or 9 minutes in Avena coleoptiles. This value corresponds quite closely to that of Wartiovaara (12) who found a half time of diffusion of D_2O of 0.8 minute for Tolypellopsis. Thimann and Samuel (11) measured the rate of diffusion of tritium hydroxide (THO) out of previously incubated potato disks and found that disks of less than a millimeter in thickness had a half time of diffusion of THO of less than 1.0 minute. The data of figure 1 show also that if roots are pretreated with 5×10^{-4} M 2,4-DNP for 4 hours, the permeability is decreased, the half time increasing to 1.1 minutes. This treatment resulted in the death of Avena coleoptile segments (7) but not of *Vicia Faba* root segments, as can be shown by the fact that root cells retain the property of behaving as osmometers during the time involved. The loss of differential permeability of the cellular membrane is the only certain criterion of death of cells in such studies as these.

In order to test the effect of killing by heat, to produce conditions similar to those used by Kramer (4), and by Renner (8), root segments were heated for 30 minutes at 80° C in water. Figure 2 shows that permeability is increased about 6 times by heating. This indicates, as claimed by Kramer, that the

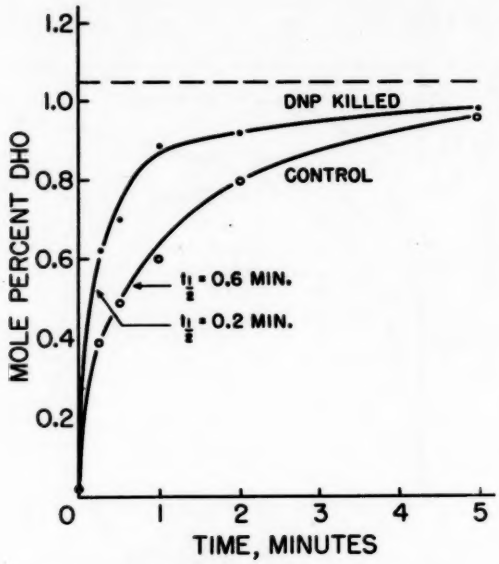

FIG. 3. Effect of killing by 1×10^{-3} M 2,4-DNP pretreatment on subsequent inward diffusion of DHO into *Vicia Faba* root sections. External solution concentration indicated by horizontal line. Half-times indicated by arrows. 0.005 M KH_2PO_4 pH 5 throughout.

primary barrier to water movement in root systems lies in the living cytoplasm of the roots.

It may be questioned whether killing by immersion in hot water is a satisfactory method of determining the relative resistance of cytoplasm and cell walls to water movement because hot water might extract pectic substances from the walls and increase their permeability. Killing with an inhibitor such as DNP would be more satisfactory because it affects only the protoplasm, hence an experiment was performed with 10^{-3} M DNP.

Root segments were pretreated with DNP for 6 hours. At the end of this time it could be seen with the microscope that root cells were not plasmolyzed by 0.8 M mannitol and they were regarded as dead. Figure 3 shows that the tissue killed with DNP also was much more permeable to water than living root segments. The half time of diffusion for the DNP-treated tissue was 0.2 minute, compared to 0.1 minute in roots killed by hot water. The higher permeability of the heat-killed tissue may be caused by extraction of pectic materials from the cell walls in hot water. The results obtained by the two methods are sufficiently similar, however, to justify the conclusion that the primary barrier to water movement in *Vicia Faba* roots is indeed the living cytoplasm.

DISCUSSION

It has been shown that living *Vicia Faba* roots are highly permeable to water and that death results in a marked increase in permeability to water as measured by DHO diffusion. Killing the roots by immersion in hot water increased permeability more than killing with DNP. Use of DNP as a killing agent seems preferable because it eliminates any possibility of extracting cell wall constituents, but the errors introduced by heat treatment apparently are not serious in studies of water permeability.

The results presented here agree with those of Brouwer (1) and Kramer (4) who found that killing roots greatly increased their permeability to water. These results also support their conclusion that the cytoplasm of the root cells is the primary barrier to water movement into the roots. Waartiovaara (12) reached a similar conclusion from experiments with single cells of Tolypellopsis.

Avena coleoptiles differ from roots in being much less permeable to water and showing a much smaller increase in permeability after death. This indicates that the walls of Avena coleoptiles present a greater barrier to water movement than the cytoplasm, a situation just the opposite of that existing in *Vicia Faba* roots. Apparently the internal walls play an important part in this resistance, because Ketellapper (3), using 2.5-mm Avena coleoptile segments, obtained half times of diffusion which were approximately half those obtained by Ordin and Bonner (7), using 5-mm segments, indicating that cutinized epidermal cells are not the only barrier to the entrance of water.

The examples of decreased permeability to water caused by inhibitors which have been reported (1, 9, 10) were verified by this investigation, although not to the extent found by others. Brouwer (1) found that KCN could reduce water uptake by 90 %, but this occurred at very high concentrations of the inhibitor. DNP caused a slight depression of permeability similar to that reported here. In both cases the depressing effect might have been due to effects on salt secretion, rather than on water movement as such. Brouwer thinks that high KCN may make cytoplasm more resistant to water flow in some unexplained manner. In any case, it is generally agreed that the effects on permeability to water which are exerted via the cytoplasm do not necessarily indicate the existence of active water uptake mechanisms.

The high values of permeability to water found by Wartiovaara (12), Thimann and Samuel (11), and the present authors, by means of the isotope method, agree with the values found by Huber and Höfler for algae (2), using osmotic methods of determination. The work of all of these authors clearly supports Levitt's (5, 6) calculations which show that there is small likelihood of the occurrence of active or non-osmotic water uptake in higher plants, because plant cells are too permeable to water for it to occur.

SUMMARY

The permeability of *Vicia Faba* roots to water was measured by the rate of diffusion of deuterium hydroxide (DHO). The half time DHO diffusion into living root segments was 0.6 minute. The half time for roots killed by DNP was 0.2 minute and for roots

killed by immersion in hot water was 0.1 minute. A lower, non-lethal concentration of DNP decreased root permeability, the average half time being 1.1 minutes.

The results of this study indicate that the cytoplasm rather than the cell walls constitutes the principal resistance to water movement in *Vicia Faba* roots. These results differ from those obtained in previous studies wih Avena coleoptiles in which permeability was little increased by killing the tissue, indicating that most of the resistance to water movement exists in the cell walls of Avena coleoptiles.

The DHO analyses were carried out by Mr. Maurice Frech of the Analytical Service Department of Consolidated Electrodynamics Corporation. We wish to thank Mr. C. W. Bailey for his helpful counsel.

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EFFECT OF RADIATION AND GROWTH REGULATORS ON LEAF ABSCISSION IN SEEDLING COTTON AND BEAN[1]

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Many workers have reported that a number of auxin-mediated responses, including growth of Avena coleoptile sections, the expansion of leaf discs, seed germination, and the photoperiodic response apparently are interrelated closely with light (4, 6, 7, 9, 12, 13, 14, 15). All of these processes have their maximum promotion in the red region of the spectrum at 6500 Å and all are freely reversed by far-red radiation, the maximum effectiveness for reversal being attained at a wave length of about 7300 Å.

Brumfield (5) noted that curvatures are induced in the roots of timothy when exposed unilaterally to ultra-violet radiation and suggested that the effect of ultra-violet is mediated through the inactivation of auxin. Bawden and Kleczkowshi (2) reported that ultra-violet injury to higher plants could be counteracted by visible light and Tanada and Hendricks (16) demonstrated photoreversal of ultra-violet damage of soybean leaves; the photoreactivation was temperature dependent and most effective at wave lengths shorter than 4500 Å.

In previous work it was found that chemical defoliation of greenhouse-grown cotton was most complete at light intensities above 3,000 ft-c and that defoliation was poor in starch depleted plants unless sugar was applied (11). Repeated field observations have indicated that the best chemical defoliation of cotton occurs on bright days of high light intensity.

Since most authorities agree that abscission is at least in part an auxin-mediated response, it was considered fundamentally valuable to investigate some of the qualitative and quantitative aspects of light as they effect the auxin relations of leaf abscission. Hendricks and Borthwick (9) have suggested that leaf abscission, interacting with other variable factors, is photoresponsive.

In the present experiments primary emphasis has been given to the interaction of light and applied growth regulators in cotyledon abscission of light-grown seedling cotton. Incidental attention has been

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given to the effect of these factors in dark-grown bean seedling-leaf abscission and to their effect on axillary bud growth of cotton.

Methods and Materials

Attempts to grow large lots of uniform cotton seedlings in the dark with cotyledonary or primary leaf petioles suitable for use gave such variable results that their use was discontinued in favor of seedlings grown in the light as described below. Black Valentine bean seedlings, satisfactory for use, were grown in an air conditioned dark room at 25 ± 1° C by the method of Downs (7). Arasan [2] treated bean seeds were planted in sterilized vermiculite contained in stainless steel growth trays. The plants were watered periodically with Hoagland's nutrient solution. The dark-grown bean seedlings were exposed to approximately 150 ft-c minutes of red light on the sixth and seventh day after planting to inhibit hypocotyl elongation and to promote leaf expansion and elongation of the leaf petioles. On the ninth day the primary leaves of all plants were debladed and the growing points were removed from all but a control lot to check further elongation. These manipulations were carried out under a green safelight. Subsequent exposures to red and far-red were given for 15 minutes.

For the cotton-seedling experiments, selfed seed of a relatively pure line, 8948 (a colchicine doubled haploid from the Deltapine strain of *Gossypium hirsutum*), were germinated in 6-inch pots and thinned to two seedlings per pot. When twenty-one days old, uniform seedlings were brought to the laboratory, the cotyledons debladed and the stem decapitated two inches above the cotyledonary node. Unpublished experiments have shown that removal of the stem tip has little effect upon either cotyledon or true leaf abscission in cotton (8). The plants were divided into lots of 12 seedlings per treatment and the distal ends of the petioles treated with either lanolin (controls) or 1% lanolin pastes of indoleacetic acid (IAA), ethylene chlorohydrin (ECH), or trans-cinnamic acid (TCA). Each experiment was repeated from three to four times on different dates giving a total of 36 to 48 plants per treatment.

Immediately following chemical treatment the seedlings were exposed to darkness, natural room-light, red, far-red, fluorescent or ultra-violet irradiations or to different irradiance levels (light intensities) as indicated below. Irradiances and light intensities were measured with a General Electric radiation meter and Weston model 603 light meter. Natural room-light averaged about 100 ft-c during the daylight hours. The red and far-red radiations were produced from sources similar to those described by Downs (6, 7) and Toole et al (17). Six 300-watt incandescent-filament lamps, with a filter of two layers of red and two layers of dark-blue cellophane, provided an effective source of far-red energy in the region of wave lengths greater than 7000 Å. A bank of daylight fluorescent lamps provided with a two-layer red cellophane filter gave an adequate source of red radiant energy in the region of 5800 to 7000 Å. Both the red and far-red light sources were adjusted to give approximately 1500 or 3000 μw/cm^2 min at the plant height in the wave-length regions of maximum effectiveness. The spectral qualities of the red and blue cellophane filters used with the above light sources have been previously characterized (6, 7, 12). Unfiltered cool white fluorescent lamps with reflectors, giving about 3000 μw/cm^2 min intensity at the plant height, were used as the fluorescent source. The ultra-violet treatments were given in an enclosed chamber fitted with two 30-watt germicidal lamps emitting radiation mostly in the 2537 Å region. The lamps were placed 150 cm above the plants. At this height the intensity of radiation was approximately 60 μw/cm^2 min. For the experiments comparing different irradiance levels (light intensity experiments), the levels below approximately 0.1 gm-cal/cm^2 min or 1000 ft-c were produced from a combination of unfiltered fluorescent and incandescent lamps with reflectors adjusted to give the desired intensities at the plant height. Natural sunlight in a shaded and unshaded greenhouse was utilized for the higher energy levels (intensities). The average maximum intensity in the shaded house was approximately 2500 ft-c or about 0.3 gm-cal/cm^2 min whereas in the unshaded house the light intensity varied from 6,000 to 8,000 ft-c at noon or approximately 0.9 to 1.2 gm-cal/cm^2 min. The length of exposure to both artificial and natural light sources was regulated uniformly to 8 hours (9 AM to 5 PM) to minimize variations in natural intensity as much as possible.

Abscission counts were made daily by applying gentle pressure to the lower side of the petioles. Percentage abscission figures for the light source (wave length) experiments with cotton are the average of four experiments, giving a total of 48 plants or 96 petioles per treatment. The irradiance data represent the mean of three experiments (36 plants or 72 petioles per treatment). Abscission for the dark-grown bean seedlings are given as the average percentage in 5 days. The regrowth in cotton was determined 18 days after treatment when the light-quality experiments were terminated. When possible the data were subjected to statistical analysis to show variability and significance.

Results

Although both light and chemicals had a significant effect upon foliar abscission of light-grown cotton seedlings, chemical treatment caused greater differences (table I). ECH and TCA greatly accelerated abscission and consistently gave the highest percentage abscission throughout. Lanolin and IAA did not promote abscission greatly; in most cases IAA inhibited the process.

The data of table I show that the rate and percentage abscission in darkness were essentially the

[2] A seed treatment fungicide, Thiram (tetramethyl thiramdisulfide), manufactured by E. I. du Pont de Nemours and Company.

TABLE I

EFFECTS OF LIGHT AND GROWTH REGULATORS ON THE AVERAGE PERCENTAGE ABSCISSION IN 12 DAYS AND THE DAYS TO 50 % ABSCISSION IN COTTON *

LIGHT TREATMENT	LANOLIN CONTROL		1 % IAA		1 % ECH		1 % TCA	
	% IN 12 DAYS	DAYS TO 50 %	% IN 12 DAYS	DAYS TO 50 %	% IN 12 DAYS	DAYS TO 50 %	% IN 12 DAYS	DAYS TO 50 %
Dark control	5	18	3	18	63	2.0	89	2.0
Room light	4	18	0	18	69	2.0	84	2.5
Red	17	16	17	18	86	2.5	80	2.5
Far-red	13	16	11	18	94	1.5	96	1.5
Fluorescent	35	16	18	18	90	1.5	100	1.5
U.V., 1.5 hrs	38	14	57	2	100	2.0	96	2.0

Values required for significance for:	% Abscission in 12 days		Days to 50 % abscission	
	Light **	Chemicals **	Light **	Chemicals **
0.01 level	2.64	2.16	0.70	0.59
0.05 level	1.69	1.62	0.53	0.45

* Each datum represents the mean of 96 petioles.
** Light × chemicals interaction is highly significant.

same as that in room-light under comparable chemical treatments. The percentage abscission of the lanolin and IAA treatments increased slightly but significantly under red and far-red radiation compared to darkness or room-light. In the ECH and TCA treatments far-red induced greater abscission than darkness, room-light or red radiation. With the exception of ultra-violet, the fluorescent source was more effective than other types of radiation in all treatments. The fluorescent source produced considerable short wave length energy as well as higher total energy than the red or far-red light sources.

Although ultra-violet at 2537 Å is not found in sunlight this available radiation source was used to show the effects of the shorter wave lengths, particularly those that cause severe injury in living cells, on abscission. For the experiment shown in table I, the seedlings received ultra-violet for only 1.5 hours and then were left in the enclosed dark chamber. In all treatments (table I) ultra-violet gave high abscission values. It was found in preliminary experiments that 3 hours of ultra-violet radiation drastically reduced abscission and 24-hour exposure entirely prevented it. No doubt this reduction is due to rapid tissue injury and death as a high rate of mortality occurred after 3 hours of irradiation and no seedlings survived after 24 hours of exposure.

To determine the effects of ultra-violet exposures shorter than 1.5 hours and the possible photoreversal of the ultra-violet effect by other wave lengths, the experiments summarized in table II were conducted. Twenty minutes of ultra-violet followed by continuous darkness increased the percentage abscission of controls from 38 % observed with one 1.5 hours exposure to 88 %, while 20 minutes of ultra-violet in combination with IAA was less effective than 1.5 hours (tables I and II). A further reduction of the abscission-inhibiting effect of applied IAA by ultra-violet exposures up to 1.5 hours suggests IAA inactivation. Ultra-violet plus far-red appear complementary to increasing abscission, whereas the promotion of abscission by ultra-violet is reduced when ultra-violet is followed by continuous red or fluorescent irradiation. This reduction possibly demonstrates a photoreversal of the ultra-violet effect upon abscission similar to that noted by Tanada and Hendricks for damage to soybean leaves (16).

Pre-treatment of cotton seedlings with room-light, red and far-red for 20, 40, and 60 minutes followed by darkness was compared to continuous illumination

TABLE II

COMPARISON OF CONTINUOUS IRRADIATION TO 20-MINUTE ULTRA-VIOLET PRETREATMENT REGIMES ON AVERAGE PERCENTAGE ABSCISSION IN COTTON IN 7 DAYS *

LIGHT TREATMENT	LANOLIN CONTROL	1 % IAA	1 % ECH
Continuous dark	2	0	73
20 min U.V.–Dark **	88	39	100
Continuous red	0	0	96
20 min U.V.–Red **	56	18	93
Continuous far-red	3	5	98
20 min U.V.–Far-red **	92	63	100
Continuous fluorescent	7	4	100
20 min U.V.–Fluorescent **	38	47	92

Values required for significance for average % abscission in 7 days:	Light †	Chemicals †
0.01 level	2.56	1.57
0.05 level	1.93	1.18

* Each datum represents 96 petioles.
** Last treatment continuous for 7 days.
† Light × chemicals also highly significant.

under these sources and to continuous darkness. Compared to continuous darkness the short pre-treatments were essentially without effect on abscission and were less effective than the continuous irradiation treatments. Cotton petioles treated with lanolin and the growth regulators were also subjected to alternating 24-hour regimes of darkness, room light, red and far-red and compared to the corresponding continuous regimes. It was noted that alternating radiation had little effect upon the final percentage abscission. However, when the 24 hours of far-red was given first the rate of abscission in the ECH and TCA treatments was accelerated slightly compared to the other light regimes.

Based on the results of the light-intensity experiments with cotton (fig 1), abscission in the lanolin controls was promoted by increasing intensities up to 2,500 ft-c. Although abscission in the IAA treatment was reduced compared to the control, it paralleled rather closely the light-intensity effect curve of the control. ECH gave high abscission at all intensities below 2500 ft-c but above 2500 ft-c abscission was retarded as it was in the lanolin and IAA treatments. Light intensities of 6,000 to 8,000 ft-c significantly reduced abscission in all treatments. The effects of high light intensity may be indirect in that high light promotes rapid tissue dehydration and cellular inactivation (8).

The percentage abscission of debladed primary leaves of dark-grown bean seedlings are presented in table III. Removal of the apical meristem region had no effect on abscission in the continuous-dark controls.

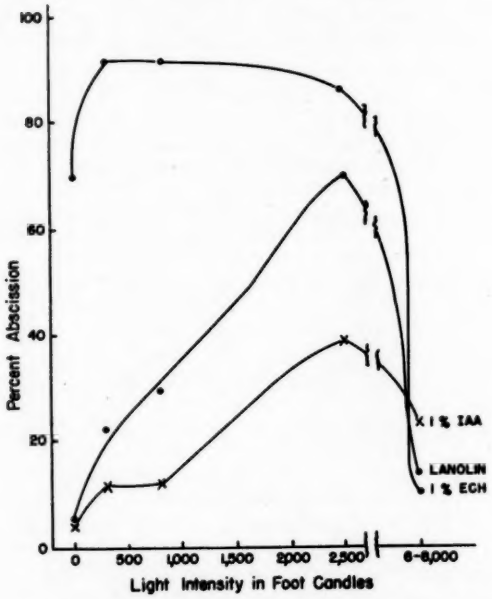

FIG. 1. Influence of light intensity on percentage abscission of debladed cotton cotyledonary petioles treated as indicated.

TABLE III

PERCENTAGE ABSCISSION OF DEBLADED PRIMARY LEAVES OF DARK-GROWN BEAN SEEDLINGS IN 5 DAYS AFTER RED AND FAR-RED IRRADIATION

LIGHT TREATMENT	NO. PETIOLES TREATED	% ABSCISSION IN 5 DAYS
1. Continuous dark (apical meristem removed)	116	12.9 ± 1.0**
2. Continuous dark (apical meristem intact)	114	12.3 ± 1.5
3. 15 min Red–Dark *	94	19.1 ± 0.8
4. 15 min Far-red–Dark *	128	25.0 ± 2.0
5. 15 min Red–15 min Far-red–Dark *	104	16.7 ± 1.0
6. 15 min Far-red–15 min Red–Dark *	94	19.1 ± 0.8

* Dark continuously after light pretreatments.
** Standard error.

The amount of abscission in the far-red treatment was approximately twice that of the controls, but the increase is insignificant compared to the effects of ECH or TCA (table I). Although the results suggest that the abscission-promoting effect of far-red is photoreversible by red radiation, the small differences in abscission make it difficult to conclusively demonstrate reversibility.

The rate of regrowth from lateral buds of cotton in the field after chemical defoliation has a profound influence on the efficiency of machine harvesting. Therefore, it was of interest to check the effect of light upon axillary bud growth. Table IV shows that red light has a pronounced stimulating effect on axillary bud growth regardless of chemical treatment. Also, red-light treatment caused the seedlings to be greener and more vigorous. This agrees with the promoting effect of red radiation on coleoptile section elongation, and on the stem and leaf growth of etiolated seedlings (7, 9, 12, 14). The control plants when placed under approximately 3000 μw/cm^2 min of fluorescent light gave a lower percentage of forced buds than when placed under about 1500 μw/cm^2 min of red light but they had a greater weight of regrowth. When grown under approximately equal energy levels, both percentage forced buds and weight of regrowth per plant was higher under the red source. This suggests that wave length as well as total energy is important in the initiation and growth of lateral buds. Darkness and ultra-violet entirely prevented regrowth in all treatments while low intensity room-light and far-red either prevented or reduced the initiation and growth of axillary buds (table IV).

DISCUSSION

Most, if not all, morphogenetic responses of higher plants are controlled by light reactions which are largely independent of photosynthesis. As recently reviewed by Downs (7), and Hendricks and Borthwick (9), an increasing list of light controlled responses have been found to be most effectively regulated by energy in the red region of the spectrum and are freely reversible by far-red radiation. All of the

photomorphogenetic responses investigated to date appear to be controlled by a common basic mechanism (7). Liverman and Bonner (12) have suggested that several of the red–far-red reversible plant responses can be interpreted in terms of a morphogenetic photocycle wherein auxin and light interact in some way to control the response.

It is well established that auxin will inhibit abscission; some believe that auxin is the principal endogenous regulator (1, 10) of abscission, i.e., will both promote or inhibit the process. Our findings that removal of the apical meristem does not greatly affect abscission in the bean are contrary to the results reported by Jacobs for Coleus (10). Removal of the apical meristem in cotton is also without effect in this regard (8). In view of other plant responses to auxin which are influenced by red–far-red energy, it would appear logical that abscission would likewise be influenced by these wave lengths. Although light has been shown in the present study to significantly modify abscission, the responses to light have not been as great as the effects of chemical treatments. Wave lengths other than ultra-violet promoted abscission only slightly. Since leaf injury (whether mechanical, chemical, or biological) is known to promote the abscission process, it is believed that the promoting effect of ultra-violet on the lanolin-control plants represents largely an injury mechanism. The relatively high amount of abscission in the IAA treatment involving ultra-violet suggests that auxin inactivation or immobilization of its effect is likewise a factor. Twenty minutes of ultra-violet followed by continuous red or fluorescent light reduced the abscission-promoting effect of ultra-violet. This possibly demonstrates a photoreversal, similar to that noted by Tanada and Hendricks (16) for ultra-violet damage to soybean leaves.

With dark-grown bean seedlings the slight abscission stimulating effect of far-red energy was reduced by red radiation and suggests, but does not prove, the reversal of a single reaction as noted for other responses (7). If a red–far-red reaction is functioning in abscission it probably contributes only a minor part to the total abscission process; the promotion of leaf abscission appears to be more directly controlled by other factors.

Although the maximum light intensity for promoting abscission in cotton was not determined, abscission of debladed petioles was inhibited above 6,000 ft-c, probably indirectly through too rapid tissue dehydration and enzyme inactivation. On the other hand, the abscission-promoting effect of high light intensity upon the less toxic chemical defoliants observed in field plantings is believed to be associated with acceleration of chemical injury and slight leaf dehydration since both favor successful cotton defoliation. Brown and Hyer (3) studied the effects on defoliation of various lengths of darkness a) prior to, b) prior to and immediately following, and c) after the application of defoliants and IAA in three varieties of cotton and compared these treatments to similarly treated plants under normal light-dark regimes. It was noted that the "darkness" treatments reduced defoliation significantly compared to the controls; the longer periods of darkness gave the greatest reduction in defoliation. The inhibiting effect of IAA on abscission was maintained regardless of light treatments. Jacobs (10) has reported that shading can speed abscission in Coleus.

The present results support the belief that the effects of light upon abscission are mostly indirect in that they serve to reduce the inhibiting action of auxin. The lack of a pronounced promoting or photoreversal reaction to red–far-red–auxin treatments, as found for some other morphogenetic responses, suggests that abscission is not solely an auxin-mediated

TABLE IV

EFFECTS OF LIGHT* AND GROWTH REGULATORS ON AXILLARY BUD GROWTH OF COTTON AS PERCENTAGE FORCED BUDS AND AVERAGE FRESH WT IN MG/PLANT AFTER 18 DAYS

TREATMENT	LANOLIN CONTROL		1 % IAA		1 % ECH		1 % TCA	
	% BUDS	MG	% BUDS	MG	% BUDS	MG	% BUDS	MG
Roomlight	0	0.0	10	2.4	0	0.0	0	0.0
Low ** red	48	37.5	22	13.3	42	15.8	21	7.5
Low ** far-red	0	0.0	0	0.0	20	11.2	0	0.0
High † red	49	43.3	52	128.5	51	43.8	40	77.5
Fluorescent †	35	38.3	50	153.3	15	25.0	40	70.5

	Values required for significance for:			
	% Forced buds		Wt of regrowth (mg/plant)	
	Light †	Chemicals ††	Light †	Chemicals ††
0.01 level	2.17	1.95	1.74	1.56
0.05 level	1.63	1.46	1.30	1.17

Each datum represents 48 plants.
* Data for dark and ultra-violet treatments omitted as regrowth did not occur in these treatments.
** Approximately 1500 $\mu w/cm^2$.
† Approximately 3000 $\mu w/cm^2$.
†† Light × chemical interaction also highly significant.

response. There is little doubt that a photoresponsive-controlling reaction is present in abscission; it is also well known that changing light conditions in the fall is a factor in inducing leaf drop of deciduous species. However, the failure of this system to completely control abscission in the present experiments possibly lies in the explanation of Hendricks and Borthwick (9) that control is expressed only when the concentration limits are met for compounds involved and that some photoresponsive reactions interact with other variable factors.

Summary

1. With light-grown seedlings, chemical treatments had a more pronounced effect upon petiole abscission than did darkness, low intensity room light, red, far-red or fluorescent light. The promoting effect of ultra-violet light upon abscission is believed to be due to injury and to IAA inactivation.

2. Both light quality (wave length) and irradiance were shown to have a significant effect upon abscission. The light-chemical interaction was also found to be significant. High light intensity significantly reduced abscission of debladed cotton petioles, probably due to rapid dehydration and enzyme inactivation.

3. With dark-grown bean seedlings removal of the apical meristem had no effect upon abscission. The slight abscission promoting effect of far-red radiation was small compared to the stimulating effect of chemicals. Reversibility of a red–far-red reaction in abscission was not conclusively proven and it appears that the red–far-red effect is only a part of the total abscission response system.

4. These experiments do not support the belief that abscission is solely an auxin mediated response.

5. Red radiation had a definite stimulating effect upon axillary bud growth in cotton whereas darkness, room light, far-red and ultra-violet prevented or reduced regrowth.

6. The promoting effect of red light upon axillary bud growth of cotton indicated an auxin-light mediated response.

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BRIEF PAPERS

CHLOROPHYLLASE IN SOYBEAN[1,2,3]

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Chlorophyllase activity has been observed in many plants, and several methods have been devised for determining its specific action in removing phytol from chlorophyll (2, 3, 4, 5, 6). Noack (3) considered that chlorophyllase has an essential role in the photo-decomposition of chlorophyll. The water-soluble chlorophyllide is, presumably, more readily oxidized in the aqueous cytoplasm by peroxide than is the non-water-soluble chlorophyll. Koski and Smith (1), working with a mutant white seedling of *Zea Mays* L., have concluded that the albinism arises from the rate of destruction of chlorophyll exceeding its rate of synthesis. In this study, a modification of Sironval's method (4) has been used to ascertain a possible relationship between chlorophyllase activity and chlorosis in a soybean mutant.

The material used was *Glycine Max* L., var. Hawkeye (courtesy of Dr. C. R. Weber, Department of Agronomy, Iowa State College) which yields yellow (Y-Y), yellow-green (Y-G), and green (G-G) leaves in the ratio 1:2:1. These were grown under natural illumination in the greenhouse during June and July and were harvested 6 weeks after planting. It was found possible to maintain the Y-Y plants by daily spraying with a 1 % sucrose solution. *Dahlia rosea* leaves, which possess high chlorophyllase activity, were used as a reference.

Greatest activity was observed in leaf tissue which, immediately after harvesting, was homogenized in 80 % acetone at 0° C. The low-speed centrifugation pellet was found to contain all the enzyme activity and this wet fraction was used as the enzyme source. Optimum incubation conditions at room temperature were found, in agreement with Weast and Mackinney (5), to be 60 to 70 % acetone in 0.067 M phosphate buffer at pH 6.2. The enzyme appeared to be unable to distinguish between chlorophyll and pheophytin, and, since the latter is less sensitive to photo-degradation, it was used as the substrate. Pheophytin *a* was obtained from crude pheophytin by chromatographic separation on a powdered sucrose column (7).

The results of a typical experiment are shown in table I. Fresh tissue from the newest, fully expanded trifolioliate leaves was weighed quickly to give an equivalent, predetermined dry weight of 50 mg and then homogenized in a small Pyrex, Ten Brock tissue grinder. The homogenate was transferred quantitatively to a 25-ml centrifuge tube and spun at approximately 1000 × g in a small centrifuge. After three resuspensions and centrifugations to remove the soluble pigments, the wet pellet was transferred to a 50-ml incubation flask with two 1-ml aliquots of buffer. Four ml of pheophytin *a* in aceton (approximately 100 mg/l) were added, the flask sealed, covered with foil to exclude light and agitated in a small, wrist-action shaker for 12 hours at room temperature.

TABLE I

RELATIVE CHLOROPHYLLASE ACTIVITY IN SOYBEAN MUTANTS AND DAHLIA *

PLANT	OPTICAL DENSITY		RATIO, (PHEOPHORBIDE a/PHEOPHYTIN a
	PHEOPHYTIN a	PHEOPHORBIDE a	
Soybean			
Y-Y	0.247	0.363	1.47
Y-G	0.406	0.225	0.55
G-G	0.320	0.237	0.74
Dahlia	0.045	0.578	12.80

* The relative chlorophyllase activity is a linear function of the ratio of the optical density (pheophorbide *a* /pheophytin *a*). Optical densities were measured at 410 mμ after 12 hours incubation at room temperature.

To assay for enzyme activity, the entire incubant was centrifuged and the supernatant plus two 1-ml acetone pellet elutions were made up to 10 ml. One ml of this was transferred to Schleicher and Schuell no. 539, white ribbon paper and chromatographed in the dark, using the descending method, with benzene-petroleum ether-acetone solvent (10:2.5:2), in an atmosphere of petroleum ether. After development, the pheophytin *a* and pheophorbide *a* bands were eluted quantitatively with acetone and made to volume. Optical densities were measured with a Beckman DU spectrophotometer at 410 mμ.

The results show no highly significant enzyme activity in any of the soybean mutants, compared with that of dahlia, and we conclude that chlorophyllase activity is not related to this type of chlorosis in soybean.

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[3] Contribution number 469 from the Institute for Atomic Research and the Department of Botany, Iowa State College, Ames, Iowa.

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A SIMPLE AUTOMATIC SHORT PHOTOPERIOD DEVICE [1]

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The writers have constructed, for greenhouse use, a very satisfactory automatic short photoperiod device which is similar in principle to the one described by Kramer (1) but differs from it in detail both as regards the type of dark box used and as regards the electrical circuit, which is somewhat simplified.

The dark box consists of a bolted frame made with Reynolds aluminum angle rods (available from building supply dealers) and covered with two thicknesses of Tico D black sateen (available from George Wood Sons & Co., Philadelphia 5, Pa.). While our box is 30 in. (75 cm) wide, 30 in. high, and 7.5 ft (2.29 + m) long, the sturdiness and light weight of this type of construction would permit construction of a box to fit any bench width and as much as 10 ft (ca 3 m) long. There are horizontal and vertical supports at the center of the box, but no cross-bracing was needed. The box is divided into two lightproof compartments by a double thickness of sateen mounted in a frame made from Reynolds aluminum sash section components, the assembly being bolted to the center supports. Aluminum bolts and nuts are used throughout. A lamp socket is attached to an aluminum cross-piece bolted across the top center of each compartment, permitting interruption of the

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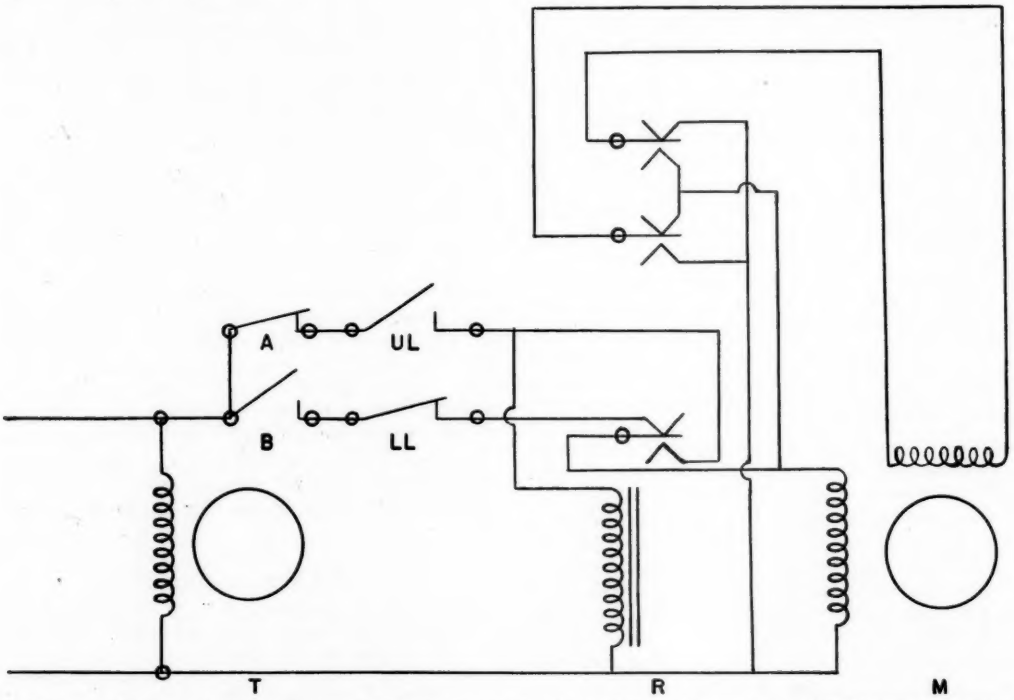

Fig. 1. Diagram of electrical circuit used in automatic short photoperiod device. A—Switch of time switch circuit a; B—Switch of time switch circuit b; M—Motor; R—Relay; T—Time switch (including time switch motor); LL—Lower limit switch; UL—Upper limit switch. The switches and relay are in their photoperiod position. The labels apply to all diagram components above them.

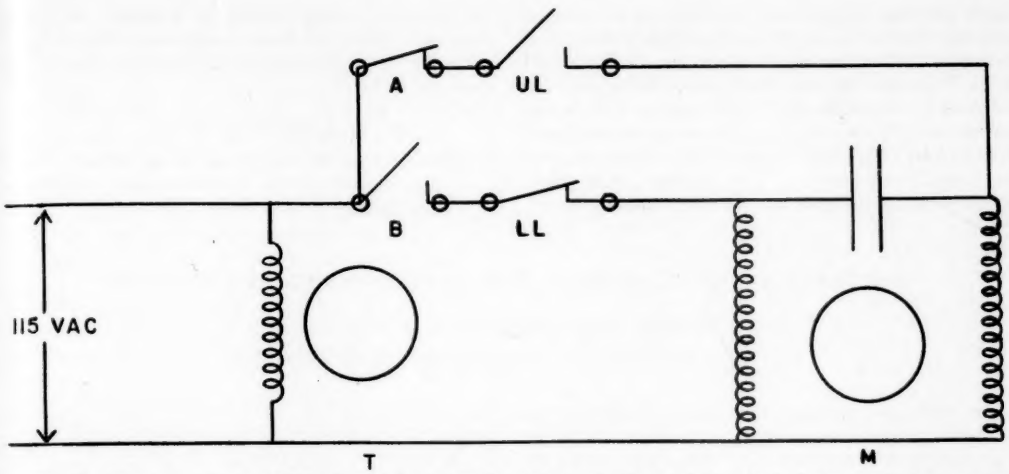

FIG. 2. Diagram of simplified electrical circuit for automatic short photoperiod devices using a capacitor motor and eliminating the relay. Labels as in figure 1.

dark period in either or both compartments. The cords from the sockets to the time switches hang free outside the box and are long enough to reach when the box is raised. The sides of the frame are covered by wrapping a continuous piece of sateen twice around the box and bolting it (with washers outside the cloth) to the frame, the excess width of cloth being laced across the box top with string. Another piece of double cloth is used to cover the top, the edges of this top cover overlapping the sides by several inches on each of the four sides. The resulting box, which fits closely inside the side boards of the bench, is light-proof to the extent that a Weston light meter reading to 0.1 ft-c failed to deflect when the box was in direct bright sunlight.

The box is raised and lowered elevator-fashion, the cord to the motor winding drum being attached to a single large eye bolt in the top center of the box. Two pulleys are used to provide proper orientation for the cord. While the steel-core plastic clothesline, used as a cord, is quite satisfactory it appears probable that heavy nylon cord would be more suitable. The box is guided into the bench by thin wires fastened vertically at each corner of the box to the bench and overhead supports and threaded through small eye bolts at the top and bottom of each corner of the box. When the box is raised to the top of the greenhouse its bottom clears the bench by 54 in. (125 cm). The shading of the experimental plants by the raised box is negligible. Adjacent benches are shaded more, but not objectionally, the net shading probably being less than with other types of automatic short photoperiod devices.

The electrical circuit is diagrammed in figure 1. The time switch (T) is a Paragon Model PS-30. The limit switches (UL, LL) are normally-closed, single-pole, single-throw microswitches of the plunger type, though the roller type might be even more satisfactory. The upper limit switch (UL) is mounted on a hinged, weighted lever arm about 8 in. (20 cm) long in such a position that the top of the box contacts the switch, opening it and so halting the upward movement. The lever arrangement prevents damage to the switch. The lower limit switch is fixed to the bench, the activating lever arm being attached to the box. The relay is a Potter and Brumfield MR14A type, 3-pole double-throw, with 8-ampere contacts.

A Boston Gear Works ratiomotor integrated motor-gearbox combination is used, with a 67.5 gear ratio and an output of 25.55 RPM. The motor is a 1/6-HP reversible R-I type with an output torque of 225 in. lbs. This torque is considerably in excess of our requirements and would provide sufficient power to raise a box of any desired size.

The daily sequence of events in the electrical circuit is as follows? 1. During the photoperiod switch A in the time switch is closed and switch B is open. The lower limit switch is closed. The upper limit switch is open because of the pressure from the raised dark box; consequently, the relay is open, and the motor idle. 2. In the evening time switch A opens and switch B closes, causing the motor to lower the box. As a result, the upper limit switch is closed and the lower limit switch opened. The relay is still open. 3. During the dark period these switch and relay positions are retained. 4. In the morning time switch B opens and switch A closes. This causes the relay to close and the motor to run in reverse. The motor thus raises the box, closing the lower limit switch and opening the upper limit switch so that the relay opens. The circuit is now again in its photoperiod status as outlined in 1 above.

The circuit described requires only one relay in place of the two used by Kramer (1). A still simpler

circuit with no relays could be achieved by using a capacitor motor such as Bodine 3810-4OH, 4-lead reversible, with attached gear box and 43 RPM and 44 in. lb. torque output. This circuit is diagrammed in figure 2. While the low output torque of capacitor motors limits their usefulness in automatic short-day devices, the use of the 1/4-in. radius drive shaft of the motor mentioned as the winding drum shaft would permit the lifting of a 176-lb. weight (neglecting friction), which would be adequate for many devices. With a 1/2-in. drum shaft the capacity would be reduced to 88 lb. and with a 1-in. radius shaft to 44 lb.

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PHOTOPERIODIC INDUCTION OF DISBUDDED PERILLA PLANTS[1]

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In 1949 Lona demonstrated that detached leaves of *Perilla ocymoides*, a short day plant, could be photoperiodically induced (1). This was shown by placing a leaf, along with a short piece of petiole or petiole and stem, in sand culture in short day conditions (14-hour nyctoperiod) for 18 days. After induction the detached leaf was grafted to a vegetative receptor plant using the procedure to be described in this paper. Within 5 weeks after grafting Lona observed macroscopic flower buds at the receptor apices, and, since care was taken to exclude both buds and roots, he concluded that the leaf blade alone was sufficient for short day induction.

Recently Carr (2, 3) extended Lona's experiments to Xanthium and soybean; with detached leaves or disbudded plants he was unable to show induction and transmission of the stimulus across a graft union. Carr concluded that a leaf blade alone was not capable of being induced and that it was necessary to have some part of a bud present during induction. This is in keeping with Gregory's theory (2) that the leaves supply the necessary substances to the buds and that the latter complete the synthesis and are responsible for the reproduction of the floral stimulus. Carr explained Lona's success with Perilla as due to the presence of bud primordia in a sufficient state of development to function in the synthesis of the stimulus.

Although Carr questioned Lona's results with Perilla he did not repeat his experiments or extend them to disbudded plants, and the purpose of this paper is to examine the question of induction of disbudded Perilla plants.

Perilla ocymoides (L.) var. *nankinensis* (Lour.) was grown from seed collected at the Botanical Garden at Parma. Prior to photoperiodic induction the plants were kept in long day conditions (photoperiod always in excess of 16 hours) until the 4th, 5th, or 6th pair of leaves, counting acropetally, had fully expanded. At this time the plants were decapitated and defoliated to a single pair of leaves (either the 4th, 5th, or 6th) and then disbudded by excavating at the nodes. The secondary axillary buds lie at the surface of the cortex at the underside of the primary axillary buds, and in order to insure their removal it was generally sufficient to remove a thin layer (1 mm or less) of the stem tissue in the axillary region. When the disbudding procedure failed the secondary buds became visible within 3 to 4 days and the plants were duly marked. In some of our control plants the original disbudding was sufficient to prevent bud regeneration for more than 3 months.

Immediately after disbudding the plants received short day induction (14- or 15-hour nyctoperiod); included in some of these experiments were plants that were partially disbudded or left intact and received short day induction and others that were completely disbudded and kept in long day conditions for a period equal to that of short day induction. Short day treatment was continued until all of the controls (intact and partially disbudded) had macroscopic flower buds (from 20 to 30 days). The induced leaves were removed with a short piece of stem and inserted into a wedge-shaped cut in the stem of the receptor plants as close to the apex as possible; the cut stem and donor leaf were then bound with raphia. During the course of the grafting procedures all cut surfaces

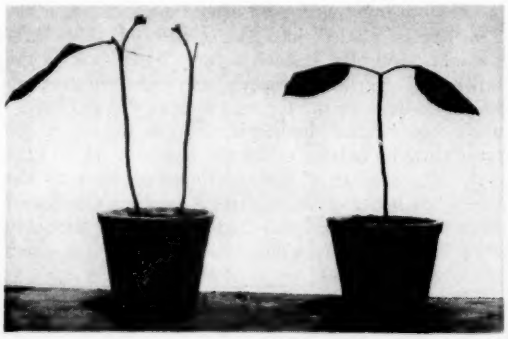

Fig. 1. *Left.* Two plants showing the wedge-shaped cut and inserted leaf. *Right.* A disbudded plant showing the 5th pair of leaves.

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were kept wet and the final raphia-bound graft was covered with a thin layer of lanolin. Prior to grafting the host plants were defoliated and disbudded except for the apical bud and leaves less than 20 mm long, and subsequently all leaves and buds which unfolded from the apex were removed. In figure 1 three plants are shown demonstrating the disbudding and grafting techniques.

For the first few days after grafting the plants were kept in a humid glass-enclosed chamber in which the light intensity was quite low (between 50 and 200 ft-c) for the major part of a 16-hour photoperiod; however, for a period of 2 to 3 hours daily the plants were exposed to natural light conditions (more than 1000 ft-c) and then returned to the glass enclosed chamber. This procedure was continued until the grafted leaves showed no signs of wilting, after which the plants remained permanently in natural conditions for a 14- to 16-hour photoperiod. Vegetative plants receiving this same treatment did not develop flowers. The temperature in the glass-enclosed chamber varied between 22 and 26° C, whereas the natural temperatures varied between 14 and 30° C. The experiments were continued until macroscopic flower buds were observed at the receptor apices.

In the summer of 1955 one of us (Lona) observed that disbudded Perilla plants could be induced; however, no quantitative data were recorded on the rate of development of the flower buds and for this reason the experiments were repeated. The results of 3 experiments are recorded in table I. The "with buds" group included all plants in which the buds had regenerated after being excised, and, therefore, the number of buds present during induction in these experiments varied from more than 10 (intact plants) to 0 (disbudded plants). For every receptor plant the number of days after grafting to the appearance of macroscopic flower buds was recorded; in all three experiments, regardless of the number of buds, flowers were observed within 30 to 40 days after grafting. That is, there was no correlation between the number of buds present during short day induction of the donor leaf and the rate of development of the flower buds on the receptor, as one might have expected if the buds were active in the synthesis of the floral stimulus.

TABLE I

RESULTS OF EXPERIMENTS ON PHOTOPERIODIC INDUCTION OF DISBUDDED PERILLA PLANTS

TREATMENT OF DONOR LVS.	No. LVS. GRAFTED	No. GRAFTS SUCCESSFUL	No. RECEPTOR PLANTS FLOWERING	% RECEPTOR PLS. FL./SUCCESSFUL GRAFTS
Short days				
Expt. A—30 SD				
With buds	10	7	6	86
Without buds	7	3	3	100
Expt. B—25 SD				
With buds	21	20	19	95
Without buds	6	6	6	100
Expt. C—20 SD				
With buds	13	13	12	92
Without buds	37	37	31	84
Long days				
Expt. A—30 LD				
With buds	6	5	0	0
Without buds	4	3	0	0

It remains to be shown that the plants "without buds" were truly lacking even microscopic bud primordia during short day induction. To this end some plants were disbudded and placed in short-day conditions. After 8 and 20 days stem sections were collected, including the axillary bud region adjacent to the remaining pair of leaves, imbedded in paraffin, and sectioned longitudinally with a microtome. The sections were stained with Heidenhain's iron hematoxylin according to the procedure described by Johansen (4) and examined under the microscope. No bud structures were apparent nor did there appear to be any abnormal meristematic growth; perhaps more important, there was no difference between the 8- and 20-day sections, thus showing that there were no regenerating tissues during the period of induction.

Regardless of the microscopic analysis it was clear that there were no "actively growing buds," in the sense in which Salisbury applied the term to Xanthium (5), present during photoperiodic induction, and this in no way affected the flower inducing property of the treated leaves. It is, therefore, highly probable that buds are not active, and certainly not necessary, in the synthesis of the floral stimulus in Perilla. These experiments lend added support to Lona's original observation on the induction of detached, disbudded Perilla leaves (1).

Salisbury (5) and Lincoln (quoted by Salisbury) have recently shown that there is a requirement for actively growing buds or young leaves for the short day induction of Xanthium. Salisbury further demonstrated that the requirement for buds could be substituted by auxin applied just after induction. He suggested that auxin in some way preserved the state of induction and that the reason why Carr was unable to show induction of detached leaves or disbudded plants may have been due to the lack of sufficient auxin. If Salisbury's interpretation is correct, the obvious explanation for the success of our experiments with Perilla is that the detached leaves or disbudded plants are capable of maintaining a sufficiently high auxin level to preserve the induced state.

SUMMARY

It has been shown, by employing a leaf-grafting technique, that disbudded *Perilla ocymoides* plants can be photoperiodically induced.

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ON THE SIGNIFICANCE OF MEASUREMENTS OF AMINO ACID INCORPORATION INTO THE PROTEINS OF CELL-FREE PREPARATIONS[1,2]

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It has been reported (4) that cell-free extracts of pea seedlings incorporate a variety of C^{14}-amino acids into their protein. The incorporation is promoted by adenosine triphosphate, by a mixture of amino acids, by magnesium and potassium ions (5), and by the nucleosides derived from ribonucleic acid (6). Amino acid incorporation is inhibited by adenosine diphosphate, by amino acid analogues, by rubidium ions, by certain purine analogues, and by treatment of the cell-free extract with ribonuclease (4, 5, 6). The properties of the incorporation system, therefore, resemble in many respects the properties of similar cell-free systems prepared from mammals (2, 3, 7) and bacteria (1).

Recently, however, the author has received several inquiries as to whether amino acid incorporation in such cell-free systems might be due to the presence of bacteria in the preparations. Although preliminary investigations indicated bacteria to be essentially absent in the disrupted seedling preparations, it is extremely important, before proceeding to other experiments, to clarify what contribution, if any, bacteria make to the measured incorporation of amino acids into protein. This report presents the results of experiments designed to investigate this question.

As noted above, amino acid incorporation by cell-free extracts of pea seedlings is promoted by adenosine triphosphate, and inhibited by adenosine diphosphate and ribonuclease. If bacteria are responsible for the observed results (4, 5) on amino acid incorporation by pea seedling preparations, then the incorporation of C^{14}-glutamate into bacterial protein should be promoted by adenosine triphosphate and inhibited by both adenosine diphosphate and ribonuclease. The effects of these substances on the incorporation of C^{14}-glutamate by suspensions of both *Azotobacter vinelandii* and a mixed bacterial culture are presented in table I. It can be seen that the three substances have no effect on the incorporation of amino acids into bacterial protein.

The reverse situation is true in the case of the effect of antibiotics on amino acid incorporation. A concentration of 5 μgm/ml of Chloramphenicol, or 1 μgm/ml of Aureomycin completely inhibits the incorporation of C^{14}-glutamate into the protein of the mixed bacterial culture. These concentrations of antibiotics have no effect on the incorporation C^{14}-glutamate into the protein of cell-free extracts of pea seedlings. Considerably higher concentrations (100 to 500 μgm of Chloramphenicol) are required for inhibition of amino acid incorporation by the pea seedling extracts. The above results, therefore, do not support the thesis that any significant part of the amino acid incorporation by pea seedling preparations is a result of bacterial growth.

Likewise, it has been reported (4) that the particulate fraction sedimenting between 10,000 and 100,000 × g has a higher specific activity (following incubation of the cell-free pea seedling extract with C^{14}-glutamate) than any other fraction. This is not

Table I

Effects of Adenosine Triphosphate, Adenosine Diphosphate, and Ribonuclease on the Incorporation of C^{14}-Glutamate into Bacterial Protein

Addition to system *	Cpm/mg protein	
	Azotobacter	Mixed bacteria **
None	1080	836
Adenosine triphosphate	1051	847
Adenosine diphosphate	1112	819
Ribonuclease	1099	825

* Experimental conditions were those described previously (4). Complete system contained 0.4 M sucrose, 0.05 M potassium phosphate buffer (pH 7.5), 0.01 M glutamate-2-C^{14} with an activity of 100,000 cpm, 0.1 mg each of a mixture of 17 amino acids (4), and the bacterial suspension containing approximately 50 mg of protein. Incubated at 38° C for 60 min. The adenyl compounds were present at 0.001 M concentration (4). Ribonuclease (1 mg/ml) was ineffective whether added at zero time or one hour before the addition of the C^{14}-glutamate. The bacteria absorbed all of the C^{14}-amino acid from the incubation medium during the 60 min incubation period.

** Prepared by inoculating a peptone-sucrose medium with dust collected from the laboratory.

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TABLE II

SPECIFIC ACTIVITIES OF CENTRIFUGAL FRACTIONS OF PEA SEEDLING EXTRACTS AND OF INTACT BACTERIAL CELLS *

CENTRIFUGAL FORCE	CPM/MG PROTEIN	
	CELL-FREE EXTRACT OF PEA SEEDLINGS	INTACT CELLS OF MIXED BACTERIAL CULTURE
500 × g for 15 min	23	850
10,000 × g for 15 min	31	876
100,000 × g for 120 min	55	893 **
Supernatant from 100,000 × g for 120 min	11	0

* Cell-free extracts of pea seedlings or a suspension of intact bacteria were incubated with C^{14}-glutamate as described in table I. The preparations were then submitted to the centrifugal forces described above and the various sediments and the supernatant assayed for radioactivity.

** This fraction contained approximately 1% of the total bacteria of the original suspension, but its specific activity is essentially the same as the sediment from 10,000 × g which contained more than 90% of the bacteria.

the case with bacterial suspensions. As can be seen from table II, each centrifugal fraction of a suspension of intact bacteria has the same specific activity, because some intact bacteria sediment at each force, even though most of the cells sediment between 500 and 10,000 × g.

Moreover, the kinetics of amino acid incorporation differ markedly in cell-free extracts of pea seedlings and in bacterial suspensions. As is evident from table III, the rate of amino acid incorporation by pea seedling extracts decreases steadily during incubation, and reaches zero after approximately two hours. In contrast, the rate of incorporation by a bacterial suspension proceeds steadily over a three-hour period.

Finally, if cell-free extracts of pea seedlings are prepared and incubated by standard aseptic techniques, the results reported previously (4, 6) can be consistently confirmed. In these cases, an aliquot of the incubation system plated out on glucose-agar at the end of the one hour incubation period, shows no evidence of the presence of bacteria. This demonstrates that the reported results (4, 6) can be confirmed in the absence of bacterial cells.

TABLE III

TIME COURSE OF INCORPORATION OF C^{14}-GLUTAMATE INTO THE PROTEIN OF CELL-FREE EXTRACTS OF PEA SEEDLINGS AND OF INTACT BACTERIA *

INCUBATION TIME (MIN)	CPM/MG PROTEIN	
	PEA SEEDLING EXTRACT	BACTERIAL SUSPENSION
30	61	615
60	85	1478
90	95	2323
120	101	3159
150	101	4044
180	101	4865

* Preparations were incubated as described in table I. Figures given refer to total radioactivity incorporated after the times indicated.

The various lines of investigation cited above, therefore, not only do not support the suggestion that amino acid incorporation by cell-free extracts of pea seedlings as reported previously (4, 5, 6) is due to bacteria, but they also provide compelling evidence against such a supposition. It should be noted, however, that the vigorous incorporating activities of bacteria, as shown in these results, constitute an ever-present danger during all metabolic studies with cell-free extracts of plants, animals, and bacteria. The desirability of some evidence of the non-participation of bacteria in results obtained in such studies is obvious. For example, comparable experiments were not performed with liver microsomes (7) or disrupted bacteria (1) during this investigation, but the dependence of amino acid incorporation in these systems on adenosine triphosphate and the strong inhibitions caused by ribonuclease (1, 7) provide evidence that the observed amino acid incorporations by such preparations likewise are due to the activities of the preparations themselves.

It is, therefore, concluded that reports of amino acid incorporation by cell-free preparations of liver (3, 7), bacteria (1), and pea seedlings (4, 6) are not due to contamination of the preparations with growing bacteria, and that no basis has been found in support of suggestions to the contrary.

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USE OF AN OPTICAL PLANIMETER FOR MEASURING LEAF AREA[1]

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The rapid, accurate measurement of area in biological materials of varying colors, textures, and shapes is of special concern to the plant scientist. The need for the measurement of leaf area, in particular, has prompted the search for newer and faster methods in recent years. In general, leaf area is an excellent indicator of the photosynthetic capacity of a plant and the measurement of such area is of value in studies in plant nutrition, plant competition, and plant-soil water relations. Leaf size or the total leaf area of a plant may also be an important consideration when studying the response of plants to growth regulators and herbicides.

Frear (1) has reviewed the laboratory methods employed in such measurements and described an instrument which he designed for the purpose. Frear's method was a simplified version of the integrating sphere technique used in photometry. His apparatus consisted of a light source and photronic cell at opposite ends of a white painted box. A ground glass plate, masked to a circular opening 18 cm in diameter, was placed midway between the cell and light source. A white, hemispherical baffle was interposed in the direct path of the light below the glass so that the response of the cell was primarily a measure of the diffused illumination from repeated scattering of light within the box. When the leaf material was placed on top of the plate it absorbed light, lowering the level of scattered illumination so that the cell response was correspondingly decreased. Since this decrease was a measure both of the area and of the effective absorption and reflection coefficients of the leaf material, it was necessary to calibrate the instrument with known areas of the particular leaf material to be employed. With highly articulated materials such as carrot leaves it was difficult to obtain known areas for calibration. Furthermore, any variation in leaf color or texture due to difference in species, weather, maturity, or time lag between picking and measurement would cause appreciable error. Accordingly, when a new instrument with larger capacity was needed in this laboratory, a basically different apparatus was developed to overcome these limitations.

The new design was made practicable by the present availability of large high-aperture Fresnel type lenses (from Special Product Sales Division, Eastman Kodak Company, 343 State Street, Rochester, New York) molded from a thin plastic sheet; these are virtually free of spherical aberration and are obtainable at low cost. With two of these lenses the light from a projection bulb was collimated into a 16-inch (40 cm) diameter beam, passed through the leaf material carried on a glass plate, and refocused on the photronic cell. Since the effective area of the cell subtended less than one percent of the solid angle seen by the second lens, a negligible fraction of the light diffused or scattered by a leaf reached the cell. With this arrangement, even such translucent materials as tracing paper are practically as effective as opaque black paper in blocking off light from the cell, so that leaf color and texture calibration is no longer necessary.

A number of refinements were incorporated into this simple scheme to improve reliability and convenience: 1) Since the Fresnel lenses accentuate the

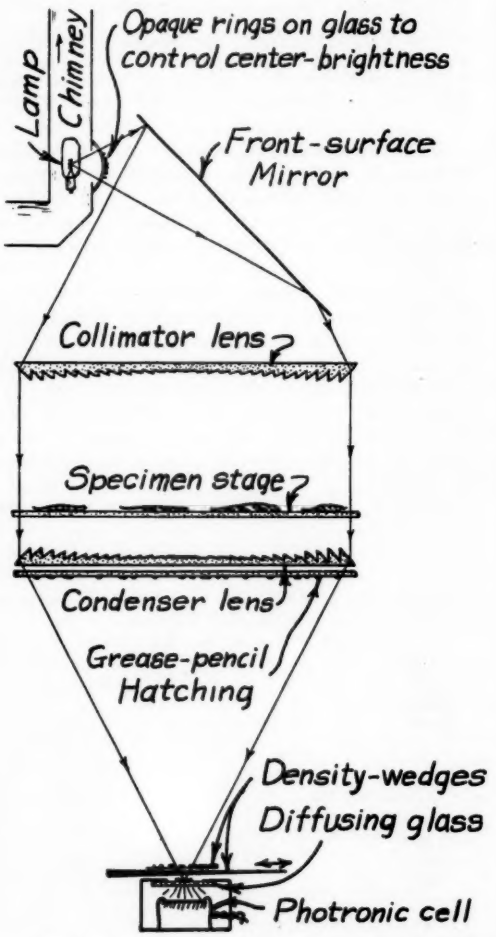

FIG. 1. Essentials of optical planimeter. A negligible percentage of light scattered by translucent leaves on the specimen stage reaches the photronic cell.

[1] Revised manuscript received September 13, 1956.

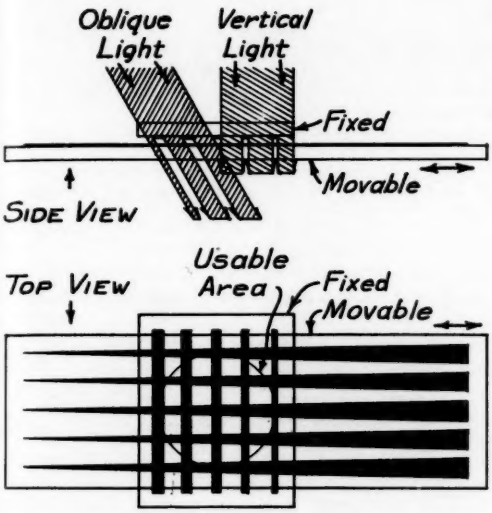

FIG. 2. "Hit or miss" type of variable-density filter. Fraction transmitted is not affected by the angle of incidence. Actual filters contain many more "spears" than are shown here.

normal tendency of a collimated beam to be brighter in the center than at the edges, compensation for this was introduced at two points as indicated in figure 1. The primary compensation was carried on a zero-power spectacle-lens blank. This blank was first covered with black paint on one side. The paint coating was then cut away cleanly to leave rings whose proper widths and spacings were computed from light response measurements. The cutting was done on a lathe using a 0.01 inch (25 mm) section of a razor blade edge as the tool. The cutting edge was held at a slicing angle by the spring tension of the blade. Control to 0.001 inch (2.5 mm) was obtainable from the cross feed head. Secondary compensation of residual non-uniformity was obtained with grease-pencil cross-hatching on a glass surface not exposed to dust or handling. Control of this was obtained by moving a hole of fixed area from place to place, hatching or erasing at each spot as needed until the photronic cell current reached a chosen fixed value. 2) In order to obtain uniform response from any photocell or photronic cell, it should be mounted behind diffusing layers to spread out the effect of local "hot spots" on the photoactive surface. This arrangement can be seen in figure 1. 3) Because of the nonlinearity of photronic cells, the illumination was always set to a constant level by means of a wedge-type density filter. As an added convenience, the latter was calibrated directly in area units. With f/0.9 Fresnel lenses, light from the edges of the field penetrates the wedges at angles sufficiently far from the vertical that conventional absorbing wedges would appear appreciably more dense to such rays than to central rays falling on the same spot. For this reason a "hit-or-miss" type of wedge-pair was devised which has the property that its average density is independent of angle of incidence (fig 2). This wedge, designed to a constant density taper so that the overlap region of the two parts will have a constant average density, was drawn out on a large scale and reduced with a camera on to glass photographic plates. 4) To combat the effect of line voltage variation, the light source was operated from a variac, the output of which is monitored by a sensitive arrangement and

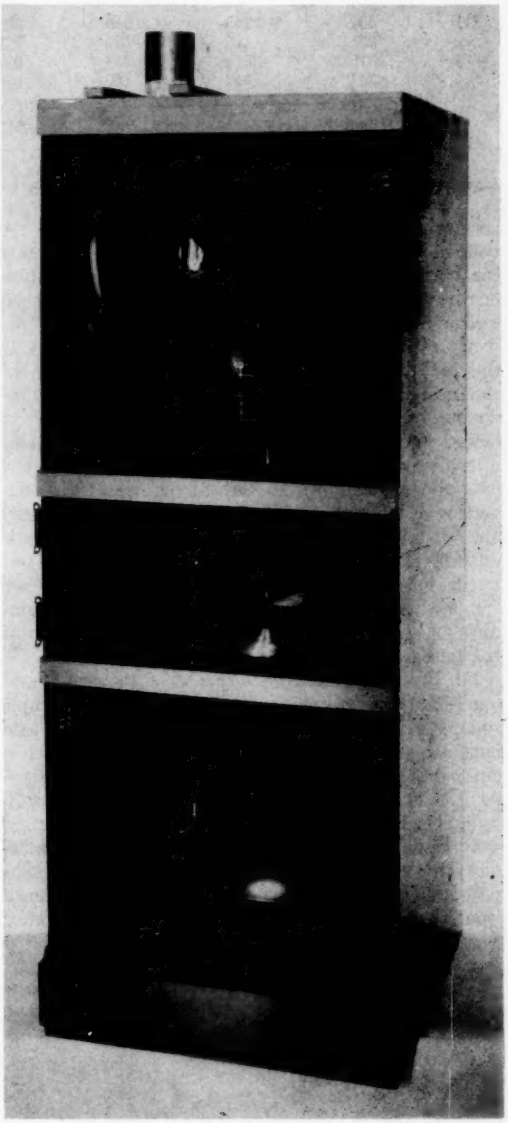

FIG. 3. Photograph of optical planimeter with front covers removed.

TABLE I

DETERMINATION BY MEANS OF A PLANIMETER OF THE LEAF AREA OF CARROT PLANTS GROWN IN COMPETITION WITH WEEDS FOR A PERIOD OF 5.5 WEEKS FOLLOWING EMERGENCE

REP.	NO. OF WEEDS/FT²							
	0		3		6		10	
	LEAF AREA IN.²	DEVIATION	LEAF AREA IN.²	DEVIATION	LEAF AREA IN.²	DEVIATION	LEAF AREA IN.²	DEVIATION
	x	$\frac{x-\bar{x}}{\bar{x}}$	x	$\frac{x-\bar{x}}{\bar{x}}$	x	$\frac{x-\bar{x}}{\bar{x}}$	x	$\frac{x-\bar{x}}{\bar{x}}$
1	502	+0.243	310	−0.292	143	−0.302	125	−0.008
2	455	0.126	130	0.458	244	0.190	129	0.040
3	318	0.213	379	0.579	186	0.093	113	0.089
4	341	0.156	140	0.417	247	0.205	128	0.032
	$\bar{x}$	C.V.(%)	$\bar{x}$	C.V.(%)	$\bar{x}$	C.V.(%)	$\bar{x}$	C.V.(%)
	404	19.0	240*	45.7	205*	25.2	124**	5.3

* Significantly different from check (no weeds) at 5 % level. L.S.D. = 142.01.
** Significantly different from check (no weeds) at 1 % level. L.S.D. = 204.23.

held within suitable tolerances during readings. A voltage-regulating transformer helps to simplify this control. (For critical problems some consideration has been given to the use of two cells in a balanced bridge; the second cell being mounted to "look" directly at the original light source as monitor. 5) If the bulb were to burn out, some readjustment of the secondary compensation cross-hatching would undoubtedly be required after the replacement was made. To avoid this, the bulb is given an indefinitely long expected life by operating it well below its rated voltage. 6) As with any instrumental conversion of information from perishable material, assurance of reliability of data should be secured. This is done by checking the null—no leaves present—at frequent intervals, and also by checking one or two standard known areas (paper squares) at the beginning and end of each work period. A photograph of the finished planimeter is shown in figure 3.

In order to obtain an estimate of the error, systematic and otherwise, readings were taken on sample squares of known area. These squares were cut into irregular pieces, and distributed at random, but without overlap over the working surface of the instrument. The sample areas used were 15, 35, and 70 square inches (ca 94, 219, 438 cm²), and 60 readings were made on each sample. The coefficient of variability for each of these known sample areas was computed. The greatest of these was 1.5 %, determined from the 70 square inch (ca 438 cm²) sample area. The deviations of the smaller areas were considerably less.

This instrument has been used to determine the total leaf area of carrot and beet plants which were grown in competition with weed populations of varying density (2). Leaf area measurements were made at regular intervals as the crops and the weeds increased in size and the competition became more intense. Representative data on the effect of varying degrees of weed competition on the leaf area of carrots are presented in table I. In all cases the coefficient of variability within the treatments was greatly in excess of that found in the planimeter. (Despite the considerable variation within the treatments, a high degree of significance was found between treatments.) It is apparent that under most conditions of biological experimentation, the error of the instrument is small enough to be regarded as negligible.

SUMMARY

The optical planimeter described above was designed to accommodate large areas and is particularly convenient for the measurement of leaf area. It is capable of rapid determination of irregular areas with high accuracy and is, therefore, suitable for use with many biological materials.

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2. SHADBOLT, C. A. and HOLM, L. G. Some quantitative aspects of weed competition in vegetable crops. Weeds 4: 111–123. 1956.

American Chemical Society Symposium: The Division of Agricultural and Food Chemistry of the American Chemical Society will hold a symposium entitled "The Trace Elements in Human, Plant, and Animal Nutrition" in Miami, Florida, April 7 to 12, 1957.

D. M. Doty will preside at the first session at which the following papers will be presented.

1. E. J. Thacker and Kenneth C. Beeson (U. S. Soil, Plant, and Nutrition Laboratory, Ithaca, New York) Occurrence of trace element deficiencies and toxicities in the United States and problems of their detection.

2. Ivan Stewart (University of Florida Citrus Station, Lake Alfred) Use of trace elements in Florida agriculture.

3. J. R. Couch (Agr. and Mech. College, College Station, Texas) Trace element research in poultry nutrition.

4. L. Seekles (University of Utrecht, Netherlands) Some aspects of the trace element problem in animal nutrition.

5. Alfred N. Meiss (Rutgers University, New Brunswick, New Jersey) Unified study of trace elements in a substrate-plant-animal complex.

Papers for a second session include the following.

6. Henry J. Koch, Jr. (Memorial Center for Cancer and Allied Diseases, New York City) Trace elements in human nutrition with reference to wide-spectrum mineral salt mixtures.

7. Arnold E. Schaefer (National Institutes of Health, Bethesda, Maryland) Seasalt in the over-all nutritional status of people in the Near East and far eastern countries.

8. R. E. Nusbaum (Atomic Energy Project, University of California, Los Angeles) Some spectrographic studies of trace element storage in human tissues.

9. Perry R. Stout (University of California, Berkeley) Cobalt, sodium, iodine, and fluorine supplies for animals through plant materials.

10. C. F. Mills (Rowett Research Institute, Aberdeen, Scotland) Comparative metabolic studies of inorganic and herbage-complex forms of copper in rats and sheep.

At a third session, the following papers will be presented.

11. C. L. Comar (Oak Ridge Institute of Nuclear Research, Oak Ridge, Tennessee) Fission products in the food and biological cycle.

12. David E. Green (Institute for Enzyme Research, University of Wisconsin, Madison) Role of trace elements in biological oxidations and oxidative phosphorylation.

13. Alvin Nason (McCollum-Pratt Institute, Johns Hopkins University, Baltimore, Maryland) Function of trace elements in enzyme systems.

14. George K. Davis (University of Florida, Gainesville) Mechanisms by which trace elements function in the animal body.—Firman E. Bear, *Soils Department, Agr. Expt. Station, New Brunswick, New Jersey.*

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